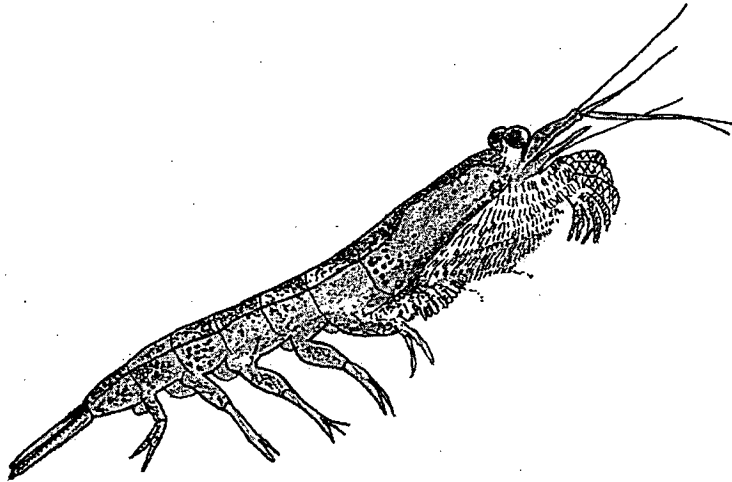


# Growth and Ageing in Antarctic krill

Growth, ageing and mortality in *Euphausia superba* Dana: new information using extractable pigments to estimate the age of krill, with implications for population modeling and the management of the fishery.

Angela Fleur McGaffin BSc (Hons)  
University of Tasmania

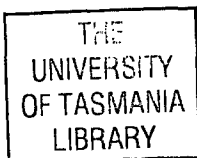


Submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

Institute of Antarctic and Southern Ocean Studies  
School of Mathematics & Physics  
University of Tasmania

June 2009

Morris  
Science  
McGAFFIN  
PhD



2009 'Shrimps and the delicate periwinkle

Such are the sea-fruits lasses love:

Ho! To your nets till the blue stars twinkle,

And the shutterless cottages gleam above!'

Bayard Taylor

*The Shrimp-Gatherers*

'...Then late the next night, I awoke in fright to a sound in the corridor  
Footsteps running, someone coming and turning the knob of my door  
I sat up in bed and hit my head but it didn't lessen the thrill  
When Angela cried "Get up – look alive, I've finally caught some krill!"'

Gordon Lindsay

from *The Glaciologist*

'The most exciting phrase to hear in science, the one that heralds new discoveries,  
is not Eureka! (I found it!) but rather "hmm... that's funny...."'

Isaac Asimov

## Declarations

1. This thesis contains no material that has been accepted for a degree or diploma by the University of Tasmania or any other institution, except by way of background information and is duly acknowledged in the thesis.
2. To the best of my knowledge and belief this thesis contains no material previously published or written by another person, except where due acknowledgement is made, nor does this thesis contain any material that infringes copyright.
3. This thesis may be made available for loan and limited copying in accordance with the *Copyright Act 1968*.



Angela Fleur McGaffin

14<sup>th</sup> June 2009

## Abstract

---

Antarctic krill, *Euphausia superba*, are an integral component of the Southern Ocean ecosystem and the target of a substantial fishery. Demographic information used to regulate the krill fishery is presently deficient, mainly due to difficulties in measuring growth and ageing using conventional approaches. This study investigates alternative techniques used to age krill, in conjunction with studies of growth and mortality.

Age-pigments were extracted from both wild caught krill and krill hatched from eggs and reared for four years in the laboratory. Wild krill collected from Antarctica were used for growth and mortality studies, and to investigate the effects of environmental variability on the accumulation of age-pigments.

Growth and mortality of krill was observed in the laboratory under food and temperature conditions that reflected the natural environment. Carapace length and eye diameter of live krill were regularly measured, and population mortality rates calculated. Control experiments were used to establish the effects of handling.

Growth curves for live krill at different temperature and food regimes were constructed over 230 days. Short-term growth (<90 days) was comparable with published data, but in the longer term (90-230 days) growth patterns reflected temperature and nutritional differences. In all conditions krill initially shrank, followed by positive growth, recovering quickly when well-fed. Growth was significantly higher and occurred earlier in krill maintained at 3°C compared with those at 0°C.

Under control conditions the average mortality rate in krill was 1 in 1000 per day. Mortality rates significantly increased when krill were either food-limited, fed phytoplankton monoculture, or fed a mixed diet, compared to the mortality rate for the control group. Mortality was not significantly influenced by temperature or handling in the long-term.

The krill populations were periodically sampled and analysed for biochemical evidence of ageing using extractable pigments. The population reared from hatching in the laboratory provided known-age krill for age-pigment calibration. Extractable pigments from the eyestalk ganglia and eyes of krill were



quantified using fluorescence intensity, and standardized against a measure of protein in each sample. Quantities of pigment at two fluorescence peak intensities of  $\lambda_{ex}=280\text{nm}$ ,  $\lambda_{em}=625\text{nm}$  and  $\lambda_{ex}=463\text{nm}$ ,  $\lambda_{em}=620\text{nm}$  correlated significantly with chronological age, and the accumulation rate of these pigments was dependent on sex. A model was developed to use pigment to predict age, which was tested against the real data. Quantity of extracted pigment predicted age better than length or eye size, which have both previously been used as proxies for age. For mature adult krill, this method can discriminate between krill of a similar size aged 2 or more years apart. Manipulation of environmental variables showed that temperature, diet and stress have significant effects on accumulation of age-pigment, which increases variance in pigment in older aged krill.

This study contributes improved understanding of growth, ageing and mortality in Antarctic krill. It has shown that age-pigments can be used to estimate age in krill, particularly if used in conjunction with other demographic information.

# Table of Contents

---

|                                                                                                                             |             |
|-----------------------------------------------------------------------------------------------------------------------------|-------------|
| <b>ABSTRACT.....</b>                                                                                                        | <b>IV</b>   |
| <b>TABLE OF CONTENTS .....</b>                                                                                              | <b>VI</b>   |
| <b>ACRONYMS AND ABBREVIATIONS .....</b>                                                                                     | <b>VIII</b> |
| <b>CHAPTER 1: INTRODUCTION.....</b>                                                                                         | <b>1</b>    |
| 1.1 <i>The problem .....</i>                                                                                                | <i>1</i>    |
| 1.2 <i>Synopsis.....</i>                                                                                                    | <i>2</i>    |
| 1.3 <i>Antarctic krill .....</i>                                                                                            | <i>2</i>    |
| 1.4 <i>Krill growth &amp; ageing.....</i>                                                                                   | <i>5</i>    |
| 1.5 <i>The krill fishery and its management .....</i>                                                                       | <i>17</i>   |
| 1.6 <i>Aim of this research.....</i>                                                                                        | <i>19</i>   |
| 1.7 <i>Research approach.....</i>                                                                                           | <i>20</i>   |
| <b>CHAPTER 2: REVIEW OF AGEING THEORY &amp; EXPERIMENTAL STUDIES .....</b>                                                  | <b>21</b>   |
| 2.1 <i>Introduction.....</i>                                                                                                | <i>21</i>   |
| 2.2 <i>Ageing theory .....</i>                                                                                              | <i>22</i>   |
| 2.3 <i>Metabolism and ageing .....</i>                                                                                      | <i>23</i>   |
| 2.4 <i>Oxidative stress and ageing .....</i>                                                                                | <i>28</i>   |
| 2.5 <i>Fluorescent age-pigments .....</i>                                                                                   | <i>29</i>   |
| 2.6 <i>Using fluorescent age-pigments to age crustaceans .....</i>                                                          | <i>31</i>   |
| 2.7 <i>Studies of fluorescent age-pigments in krill .....</i>                                                               | <i>32</i>   |
| 2.8 <i>Summary.....</i>                                                                                                     | <i>34</i>   |
| 2.9 <i>Conclusions &amp; hypotheses.....</i>                                                                                | <i>35</i>   |
| <b>CHAPTER 3: MATERIALS AND METHODS.....</b>                                                                                | <b>37</b>   |
| 3.1 <i>Overview .....</i>                                                                                                   | <i>37</i>   |
| 3.2 <i>Selection of methods.....</i>                                                                                        | <i>37</i>   |
| 3.3 <i>Experimental design.....</i>                                                                                         | <i>38</i>   |
| 3.4 <i>Krill collection &amp; aquarium maintenance.....</i>                                                                 | <i>39</i>   |
| 3.5 <i>Collection of morphometric data and image analysis.....</i>                                                          | <i>42</i>   |
| 3.6 <i>Pigment extraction .....</i>                                                                                         | <i>43</i>   |
| 3.7 <i>Measurement of fluorescence.....</i>                                                                                 | <i>44</i>   |
| 3.8 <i>Approach to statistical analyses.....</i>                                                                            | <i>50</i>   |
| <b>CHAPTER 4: DIET-RELATED MORTALITY IN CAPTIVE KRILL .....</b>                                                             | <b>53</b>   |
| 4.1 <i>Abstract .....</i>                                                                                                   | <i>53</i>   |
| 4.2 <i>Introduction.....</i>                                                                                                | <i>54</i>   |
| 4.3 <i>Materials and methods .....</i>                                                                                      | <i>55</i>   |
| 4.4 <i>Results .....</i>                                                                                                    | <i>57</i>   |
| 4.5 <i>Discussion .....</i>                                                                                                 | <i>61</i>   |
| 4.6 <i>Conclusions .....</i>                                                                                                | <i>65</i>   |
| <b>CHAPTER 5: QUANTIFICATION OF EXTRACTABLE PIGMENTS IN ANTARCTIC<br/>KRILL OF KNOWN AGE .....</b>                          | <b>67</b>   |
| 5.1 <i>Abstract .....</i>                                                                                                   | <i>67</i>   |
| 5.2 <i>Introduction.....</i>                                                                                                | <i>68</i>   |
| 5.3 <i>Materials and methods .....</i>                                                                                      | <i>69</i>   |
| 5.4 <i>Results .....</i>                                                                                                    | <i>71</i>   |
| 5.5 <i>Discussion .....</i>                                                                                                 | <i>90</i>   |
| 5.6 <i>Conclusions .....</i>                                                                                                | <i>98</i>   |
| <b>CHAPTER 6: EFFECTS OF TEMPERATURE AND DIET ON GROWTH AND THE<br/>ACCUMULATION OF AGE-PIGMENT IN ANTARCTIC KRILL.....</b> | <b>99</b>   |
| 6.1 <i>Abstract .....</i>                                                                                                   | <i>99</i>   |
| 6.2 <i>Introduction.....</i>                                                                                                | <i>100</i>  |

|                                                                                          |            |
|------------------------------------------------------------------------------------------|------------|
| 6.3 Materials and methods.....                                                           | 101        |
| 6.4 Results.....                                                                         | 104        |
| 6.5 Discussion.....                                                                      | 119        |
| 6.6 Conclusions.....                                                                     | 125        |
| <b>CHAPTER 7 GENERAL DISCUSSION.....</b>                                                 | <b>127</b> |
| <i>Summary &amp; Conclusions.....</i>                                                    | <i>133</i> |
| <b>ACKNOWLEDGMENTS.....</b>                                                              | <b>135</b> |
| <b>LITERATURE CITED.....</b>                                                             | <b>137</b> |
| <i>Appendix I: Fit of von Bertalanffy growth models.....</i>                             | <i>161</i> |
| <i>Appendix II Simulation study.....</i>                                                 | <i>171</i> |
| <i>Appendix III Logistic discriminant analysis .....</i>                                 | <i>191</i> |
| <i>Appendix IV: Multivariate analysis of factors affecting pigment accumulation.....</i> | <i>227</i> |
| <i>Appendix V: Nutritional profile of Schizochytrium sp. (Algamac-2000®) .....</i>       | <i>261</i> |

## Acronyms and abbreviations

---

|          |                                                                                |
|----------|--------------------------------------------------------------------------------|
| AAD      | Australian Antarctic Division                                                  |
| ANARE    | Australian National Antarctic Research Expedition                              |
| ANOVA    | Analysis of variance                                                           |
| BIOMASS  | Biological Investigations of Marine Antarctic Systems and Stocks               |
| BSA      | Bovine serum albumin                                                           |
| CCAMLR   | Commission for (and Convention on) the Conservation of Marine Living Resources |
| CL       | Carapace length                                                                |
| DCM      | Dichloromethane                                                                |
| DHA      | Docosahexaenoic acid                                                           |
| DM       | Dry mass                                                                       |
| DOC      | Deoxycholic acid                                                               |
| DVM      | Diel vertical migration                                                        |
| ED       | Eye diameter                                                                   |
| ELEFAN   | Electronic length frequency analysis                                           |
| EPA      | Eicosapentaenoic acid                                                          |
| FAP      | Fluorescent age-pigment                                                        |
| FFM      | Foraging-fishery model                                                         |
| GYM      | Generalised yield model                                                        |
| HPLC     | High-performance liquid chromatograph(y)                                       |
| IGR      | Instantaneous growth rate                                                      |
| IMP      | Inter-moult period                                                             |
| KYM      | Krill yield model                                                              |
| LFD      | Length frequency distribution                                                  |
| M        | Molar                                                                          |
| <i>M</i> | Natural mortality rate                                                         |
| PPM      | Parts-per-million                                                              |
| PUFA     | Polyunsaturated fatty acid                                                     |
| $QO_2$   | Oxygen quotient (consumption)                                                  |
| RMS      | Residual Mean Square                                                           |
| RMT      | Rectangular mid-water trawl                                                    |

|       |                                                         |
|-------|---------------------------------------------------------|
| ROS   | Reactive oxygen species                                 |
| RTA   | Return to Australia                                     |
| SOD   | Superoxide dismutase                                    |
| SSMU  | Small scale management units                            |
| UMCES | University of Maryland Center for Environmental Science |
| VB    | von Bertalanffy                                         |
| WM    | Wet mass                                                |
| WWE   | Wet weight of the eyes                                  |

# Chapter 1: Introduction

---

## 1.1 The problem

Currently, there is no single agreed method available with which to age krill. It is therefore uncertain how long krill live in the wild, or how many year classes exist in natural oceanic populations. Previous methods applied to solving this problem have significant limitations (see section 1.4) and to date we have no definitive answers to the question of how long krill live.

The growth and longevity of krill is a matter of appreciable international and scientific interest for several reasons. The Antarctic krill, *Euphausia superba* Dana, is a keystone species in the Antarctic ecosystem, figuring as the major prey item for many predators, including baleen whales, seals, penguins, squid, fish and seabirds (Mauchline & Fisher, 1969; O'Sullivan, 1983; Smetacek and Nicol, 2004). The ecological significance of this species is undisputed. Krill are also the target of a fishery that has persisted since the 1960s (Ross & Quetin, 1988, Kock *et al.*, 2007). Fishing for an ecologically important resource - a species that anchors a food-chain - raises the potential for conflict.

The need for balance between an ongoing fishing industry and ecological conservation has been recognised by the Commission for the Conservation of Antarctic Marine Living Resources (CCAMLR), an international regulatory body established to allow fishing to occur in Antarctic waters within ecologically sustainable quotas, taking into account the needs of dependent and related species (Constable *et al.*, 2000). Effective management of the krill fishery is based on population models that require input of biological information on krill, including estimates of mortality, growth and age parameters (CCAMLR, 2006). Therefore, without being able to age krill, and hence estimate natural longevity, age at maturity or number of reproductive year classes, it is difficult to accurately predict the level of fishing effort that the population can sustain.

## 1.2 Synopsis

This study focuses on the Antarctic krill, *Euphausia superba*. Historically, considerable research has been undertaken to understand growth and ageing in this particular species of krill because of its ecological and economic importance. Despite this large historical body of research, a successful method of reliably ageing krill has not yet been developed.

The difficulty in attempting to age krill can be attributed to the way in which krill grow, the challenge of rearing krill in the laboratory and the physical constraints of undertaking research in the environment that krill inhabit. This chapter will therefore deal with aspects of the biology of the Antarctic krill and its geographic distribution that affect growth. In particular, previous growth and ageing research will be discussed to establish the current state of knowledge. The management of the krill fishery by CCAMLR, based on an age-parameter dependent population model, is also briefly discussed and why it is important that a consistent, reliable method to age krill is established.

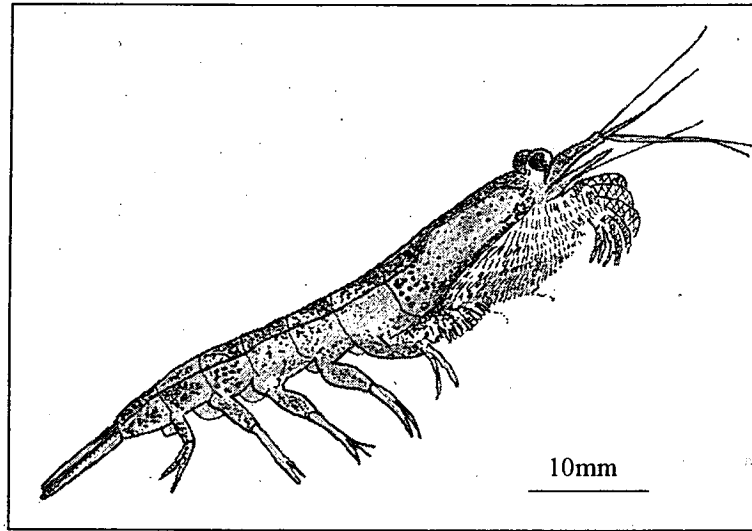
## 1.3 Antarctic krill

*Kril* was originally a Norwegian expression to describe whale food – literally ‘young fry of fish’ (Dictionary.com, 2008). Today the term ‘krill’ is used generally to describe any crustacean in the order Euphausiacea. For this thesis *krill* will be used to refer to the Antarctic krill *Euphausia superba* Dana, unless otherwise specified.

Of the 86 known species world-wide (Baker *et al.*, 1990), seven euphausiid species occur in Antarctic waters (Everson, 2000). Of these, the Antarctic krill *Euphausia superba* (Figure 1) is the largest, reaching a maximum length of 62mm (Fisheries and Agriculture Organisation of the United Nations, 2006) and is nektonic as an adult. It has the largest biomass of any krill species – estimated to be between 67 and 740 million tonnes, depending on the estimation technique used (Siegel, 2005).

Overall population size appears to be dependent on recruitment success, as opposed to being limited by predation or fishing pressure (Atkinson *et al.*, 2004; Meyer & Oettl, 2005). Recruitment has a strong environmental basis, and there is

evidence that it is linked to changes in sea ice cover from year to year (Kawaguchi and Satake 1994; Siegel & Loeb, 1995; Fraser & Hofmann, 2003). It appears that in certain areas off the Antarctic Peninsula, ice conditions suitable for good krill recruitment are becoming less common due to climate warming (Fraser & Hofmann, 2003).



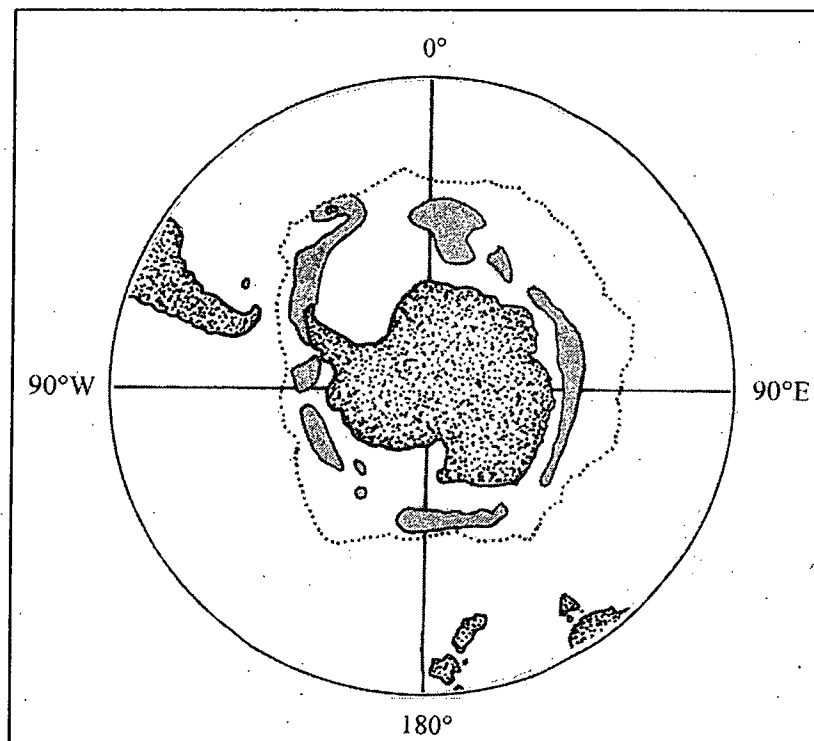
**Figure 1.** Antarctic krill, *Euphausia superba* Dana.

As with many krill species, *E. superba* forms large social aggregations, usually referred to as schools or swarms (Ritz, 1994). Hamner and Hamner (2000) correctly argue that the term swarm is often a misnomer when applied to krill, as the aggregations formed by krill at sea are usually polarised, regularly spaced and individuals swim in the same direction, quite unlike the lack of organisation associated with a swarm (Parrish & Hamner, 1997). Schools can have a density as high as 20 000 individuals per cubic metre (Kils, 2005), (estimates have been even higher; see Table 1 in Hamner & Hamner, 2000). These aggregations can be very large: one was measured covering an area of 450 km<sup>2</sup> and it was estimated that this particular school held as much as 2 million tonnes of krill (Macauley *et al.*, 1984). Very large krill aggregations are probably rare, due to the creation of an unfavourable internal environment of excreted ammonia, depleted oxygen and depleted food supplies, (Ritz, 1994; Hamner & Hamner, 2000). Nonetheless, the characteristic of forming schools, coupled with their high biomass makes krill attractive both to predators and as a fishery resource.



Krill also occupy a significant ecological niche. They form an integral trophic link between primary producers (as the main consumer of phytoplankton) and predators; krill are consumed by most of the higher order predators in the Antarctic ecosystem (Beddington & May, 1982). Usually labelled a “keystone” species for this reason, the Antarctic krill has high conservation value.

The geographical distribution of *E. superba* (Figure 2) is circumpolar between the continental shelf (Pauly *et al.*, 1996) and the Antarctic Polar Front Zone (APFZ) (Everson, 2000) and particularly the islands of the Weddell-Scotia arc. The distribution is asymmetrical (Atkinson *et al.*, 2004) with krill being largely restricted to the pack-ice zone in the Indian and Pacific sectors (Nicol *et al.*, 2000).



**Figure 2.** Broad-scale distribution of *Euphausia superba*. The dotted line indicates the relative approximate position of the Antarctic convergence. Redrawn from Miller & Hampton (1989) and Everson (2000).

The majority of the total biomass is found in the Atlantic sector of the Southern Ocean (Siegel, 2005). This wide and fluid oceanic distribution can make krill populations difficult to sample or directly observe in the natural environment. Particularly, it is difficult to revisit a population at time intervals (to monitor its characteristics) with any confidence that it is the same population, because

membership of individual krill populations may be dynamic. Winter conditions can also interfere with sampling, especially by nets, as much of the krill population is probably located under sea ice (Brierley *et al.*, 2002). As a consequence, much of what is known about krill is based on summer research and on laboratory studies (Nicol, 2000; Siegel & Nicol, 2000).

There is no doubt that *E. superba* is the most significant Southern Ocean euphausiid in terms of its ecological role and economic potential. In being a valuable resource, however, there are obvious grounds for conflict and any harvesting must be carefully managed. Management requires knowledge of the life history - particularly growth and ageing - of the animal, and its interactions within the ecosystem that is to be protected.

## 1.4 Krill growth & ageing

### *Growth*

Growth of krill is determined by the cyclic production and loss (moult) of the exoskeleton, which facilitates the basic functions of movement, protection and support in arthropods (Horst & Freeman, 1993). Growth is stepwise, with apparent growth (change in actual size) taking place at ecdysis – the point at which moulting takes place. During the period between moults, known as the intermoult or anecdysis (Carlisle & Knowles, 1959), krill consume food which is principally converted to muscle, lipid stores, other tissues and energy (Persinotto *et al.* 2000; Meyer *et al.*, 2002). The mass of an individual generally increases until it can grow no more within the constraints of the exoskeleton, at which point ecdysis occurs, although the timing is generally determined by other physiological-dependent variables such as temperature (Buchholz, 1991). Ecdysis is a 5-step process: the krill takes in a large quantity of water with which it can increase its apparent size, a soft new integument is produced, reusable substances such as ions are reabsorbed from the old shell, the old cuticle separates from the soft underlying tissues (apolysis) and then the krill escapes from the old exoskeleton, or exuviae (Buchholz, 1982; Horst & Freeman, 1993). The frequency of moulting decreases with age (Miller & Hampton, 1989; Mauchline, 1985), presumably due to an energetic conflict between allocation of resources for reproduction and growth.

Unlike other crustaceans, however, krill do not have a terminal moult (Rosenberg *et al.*, 1986), and can continue to moult throughout life, thereby adapting adult body size to environmental conditions.

Growth can be affected by a number of factors, biotic and environmental. Biological (primary) influences on the growth of krill include food availability, parasite infection, predation, effects of schooling behaviour, size, maturity stage and gender. Environmental, or abiotic, factors are also discussed, although mostly these have secondary influences on growth. Environmental factors might determine krill distribution in relation to food, for example, and therefore relate back to biological processes. As these influences are all integral to understanding krill growth, discussion of these factors follows. Shrinkage of krill is dealt with in the following section.

### Biological influences

1. The type and quantity of food available is perhaps the most obvious factor driving krill growth (McClatchie, 1988; Ross *et al.*, 2000; Fach *et al.* 2002). Food availability and nutritional value is of particular interest as krill appear to be flexible in diet, and eat whatever food is available. Krill have been reported eating phytoplankton, sea ice algae, zooplankton, marine snow, faecal pellets, moults and other krill (Kawaguchi *et al.*, 1986; Marschall, 1988; Daly, 1990; Daly & Macaulay, 1991; Nordhausen *et al.*, 1992; Cripps & Atkinson, 2000; Hamner & Hamner, 2000). Krill can even sustain long periods of starvation (Ikeda & Dixon, 1982; Buchholz, 2003). If several starved krill are kept together they will resort to eating moults and other krill that are still alive, making it difficult to undertake starvation experiments on groups (personal observation). Krill have even been reported sequestering balls of food to consume away from other hungry krill and fighting over, and stealing these food balls from one another (Hamner & Hamner, 2000). Food availability is seasonal, and each type of food provides different lipids and proteins required for growth. Krill are probably food limited in winter (Siegel & Nicol, 2000) when periods of low or negative growth have been reported (Quetin *et al.*, 2003; Kawaguchi *et al.*, 2006), and greatest growth rates are recorded during the spring

phytoplankton bloom (Ross *et al.* 2000). As food quantity and quality change during the course of a year, and from one region to another, it can be expected that krill experience a range of natural growth rates, and indeed this seems to be the case (see section below: 'measuring growth'). A dietary study on another species of krill, *Nyctiphanes australis*, showed that regardless of diet, siblings developed asynchronously by as much as 25%. Diet did not significantly influence larval growth rates, and the authors argue that genotype may be the important determining factor for larval growth (Haywood & Burns, 2003). Whether this is the case for Antarctic krill is yet to be examined. How food quality and quantity effects growth is further investigated in this study.

2. The size and maturity stage of krill is related to growth rate (Kils, 1982; Rosenberg, 1986; Pakhomov, 1995a; Kawaguchi *et al.*, 2006), so growth rates must be reported in the context of the size and apparent maturity of krill: this is because metabolism is dependent on body size (e.g. Kils, 1982). Growth is fastest in small krill, with incremental growth becoming smaller and inter-moult period (IMP) becoming longer as krill get larger and presumably older (e.g. Rosenberg *et al.*, 1986; Siegel, 1987; Candy & Kawaguchi, 2006). Sexual maturity is also relevant, as some resources must be diverted from growth to reproduction. Siegel & Nicol (2000) give a comprehensive summary of reported growth rates for different size krill, some of which is reproduced in the section "measuring growth" below.
3. Gender is significant to growth and mortality. Males and females appear to use different growth strategies in the reproductive season. Males grow faster ( $0.119 \text{ mm d}^{-1}$  compared with  $0.063 \text{ mm d}^{-1}$  for females of the same initial size, in January in the Indian Ocean sector) (Kawaguchi *et al.*, 2006) and reach larger sizes, apparently at the risk of increased mortality, (Virtue *et al.*, 1996). Females, in contrast, appear to maintain energy reserves and put energy into egg production. Subsequently, Kawaguchi *et al.* (2006) warn that growth rate estimates based on length frequency distributions (LFDs) that do not take into account gender differences may be fundamentally flawed.

4. Schooling behaviour confers protection from predators, provides access to mates, offers swimming and energetic benefits, and facilitates group foraging (Parrish & Hamner, 1997). It can also increase competition for food and exposure to disease and parasites. The advantages and disadvantages associated with being in a school are integral to the life history of krill: “schooling behaviour affects absolutely every aspect of the biology of krill after metamorphosis from furcilia VI” (Hamner & Hamner, 2000), and must therefore be an important consideration in determining growth. There is evidence that moulting may be synchronous in some swarms of krill, which may be a result of the of the moulting process affecting the swimming speed of individual krill, which would select for krill at similar moult stages (Buchholz *et al.*, 1996). As Ritz (2002) points out, social behaviour ought to be taken into account when examining the growth of krill, as schooling has direct effects on feeding and energetics.
5. Predation may play a role in growth. Alonzo & Mangel, (2001) predict that even under a positive energy budget, krill may shrink in response to predation risk. If predation risk is size dependent (Hill *et al.*, 1996), Alonzo & Mangel (2000) argue that krill may shrink between reproductive events to reduce predation. They also postulate that krill experience a trade-off between avoiding risky feeding habitat and growth and survival. Ritz (2002) counters this by suggesting that because krill use schooling behaviour as an anti-predatory defence, predation is more likely to affect the size of a school than the size of individual krill. Alonzo & Mangel (2002) defend their original model: “There is no reason to believe that swarming and shrinking are mutually exclusive responses to predation or that swarming reduces size-dependent predation risk.” Additionally, krill distribution in lower food environments (oceanic versus shelf) may represent a trade-off of reduced growth-rate with reduced risk of predation (Atkinson *et al.*, 2008).
6. Finally, parasite load is evidently an important consideration when it comes to krill growth (Takahashi *et al.*, 2004). Krill have been reported to be

infested by a variety of parasites, including epizoic ciliates (Stankovic *et al.*, 2002; Tarling & Cuzin-Roudy, 2008) and gregarines (Takahashi *et al.*, 2003; 2004). Parasites can have benign through to lethal effects. Gómez-Gutiérrez *et al.* (2003) report a mass mortality of *Euphausia pacifica* caused by parasitic infection. Most commonly however, the parasites merely utilise nutrients that would otherwise be used by the krill, and therefore have an effect on condition and growth. Gut parasites may even be beneficial, enabling krill to utilise a wider range of food sources (Kawaguchi & Toda, 1997). Parasite infestation has been correlated with krill size (Takahashi *et al.*, 2004), moult-frequency and age (Tarling & Cuzin-Roudy, 2008) but it is unclear whether infection affects krill size or vice versa.

## Environmental influences

Environmental (abiotic) factors are also important and have marked effects on the growth of animals in a polar environment. Water temperature, light regime, oceanography, and ocean chemistry all have significant effects on krill growth.

1. The light regime, which influences seasonal food availability, vertical migration in the water column, moulting, feeding activity and metabolism, is a significant external factor governing the growth and maturation of krill (Quetin *et al.*, 2003; Teschke *et al.*, 2007, Teschke *et al.*, 2008). The number of hours of daylight varies over the region in which krill are distributed (with latitude) from 0 to 6 hours in winter to 18 to 24 hours in summer (Smith & Sakshaug, 1990) and this can vary greatly over the geographic range of krill. As discussed above, krill depend largely on phytoplankton and sea-ice communities for food. Primary production is driven largely by sunlight and is therefore near zero in winter and as high as  $500 \text{ mg C m}^{-2} \text{ d}^{-1}$  in spring (Smith & Nelson, 1990). With so much less of their primary food available during the darker periods of the year, krill growth rates drop significantly (for example see Ross *et al.*, 2000; Quetin *et al.*, 2003). Even with high food availability, krill feeding activity and metabolism are linked to natural light cycles (Teschke *et al.*, 2007). Daily

light cycles are also important in determining growth. Krill undertake diel vertical migration (DVM) which is associated with feeding and reduced predation (Morris *et al.*, 1983; Tarling & Johnson, 2006). Food availability and predation may both be key factors determining growth (see above). Changes in daily light cycles also seem to provide important seasonal cues for moulting and sexual maturation, which are inextricably linked with growth (Teschke, 2008). For example, it wasn't until controlled 'natural' light regimes were introduced in krill aquaria that krill were successfully bred in captivity (Hirano *et al.*, 2003).

2. Oceanographic features indirectly affect growth. Temperature and salinity, stratification of the water column and currents can affect the distribution of adult krill (Murphy *et al.*, 1998; Trathan *et al.*, 2003), particularly in relation to food; and Quetin *et al.* (2003) demonstrated that sea-ice cover can be important in determining larval growth rates, probably because this has an effect on winter food availability. Egg sinking rates and larval ascent rates are also affected by oceanographic characteristics (Marschall & Hirche, 1984), which can therefore influence growth by the amount of time it can take for a larval krill to reach surface conditions of light, temperature and food. The relationship between krill distribution and oceanic features is complex [see Siegel (2005) and Nicol (2006) for a full discussion]. Sea ice, oceanography and nutrients are inextricably linked as factors governing primary production, which is probably the driving force behind krill distribution and growth.
3. Chemicals in the water are also important for determining growth and these fall into four classes: essential ions, acidity (pH), biogenic chemicals and toxic chemicals. Firstly, essential ions such as calcium, copper, potassium and sodium are needed for metabolic processes such as the formation of oxygen-carrying haemolymph (copper) or exoskeleton strengthening (calcium), and are periodically lost in shed exuviae (Nicol *et al.*, 1992). These ions are required for normal growth as they perform critical roles in metabolism. There is very little published information about where krill obtain essential ions; food or seawater are the presumed sources. One

study, however, examined the sourcing of fluoride, and it appears fluoride is absorbed from the water rather than being consumed in food (Nicol & Stolp, 1991). As marine crustaceans do not rely on calcium stores for remineralisation of the exoskeleton after moult (Roer & Dillaman, 1984), calcium must also be sourced from the environment. The crab *Carcinus maenas* apparently relies on seawater to source calcium (Nicol *et al.*, 1992), and it is probable that krill may also. Secondly, the pH or acidity of seawater is likely to affect krill, particularly with increasing acidification of the Southern Ocean as a result of climate change (Fabry *et al.*, 2008). Thirdly, biogenic chemicals - such as hormones from surrounding krill in a school - may have a direct effect on the growth physiology of krill, for example as a possible cause of synchronous moulting (Buchholz, 1991). This relates back to the life history of krill being inextricably linked to its life in social aggregations (see above). Finally, toxic chemicals of anthropogenic source, which include insecticides (DDT; Rakusa-Suszczewski, 1981), petrochemicals (Cripps & Priddle, 1991) and heavy metals (Yamamoto *et al.*, 1987), may have sub-lethal effects on growth. Although these chemicals have been found in krill, whether they effect krill growth and health remains untested, but seems likely.

4. Perhaps the most obvious abiotic influence on krill growth is temperature, which affects metabolic processes (Fach *et al.*, 2002) as well a seawater viscosity. Krill inhabit water that ranges in temperature from -1.8°C under sea ice to 5°C around the subantarctic islands of the Weddell Scotia arc (Quetin *et al.*, 1994). Temperature differences are very likely a major cause of different estimates of growth rate and production for different regions of the Southern Ocean. In laboratory experiments, water temperature significantly effects growth rate and intermoult period (IMP) (e.g. Poleck & Denys, 1982; Nicol & Stolp, 1991; Buchholz, 1991; Tarling *et al.*, 2006) - increasing temperature apparently causes decreasing IMP and increased growth rate. Increasing sea-water temperature above 2°C, however, may increase IMP (and reduce growth rate) for mature female krill (Tarling *et al.*, 2006), and 0.5°C may be an optimum temperature for growth (Atkinson *et al.*, 2006). The viscosity of seawater (which is affected by



seawater temperature) may be most important for the larval stages, which are much more affected by viscous, rather than inertial, forces (Nicol, 2003). It is clear that temperature is fundamental in determining krill production. The effects of temperature on krill growth will be further investigated in this study.

### *Shrinkage*

The main food source for krill is phytoplankton, which is only widely available in the spring and summer months. Questions have been raised about how krill survive winter, as phytoplankton are mostly absent from the water column during this time. It is postulated by some researchers that krill might starve during this period (Ikeda & Dixon, 1982; Shin & Nicol, 2002), and Ikeda & Dixon (1982) did in fact show that krill could survive long periods (211 days) without food by using body reserves and shrinking.

Krill apparently lose muscle mass, but not muscle cells when they shrink (McGaffin *et al.*, 2002). Muscle atrophy occurs naturally as part of the growth cycle in Crustacea (Mykles & Skinner, 1985), allowing the individual to withdraw bulky muscular parts from the old exoskeleton at ecdysis. Atrophied parts are restored at a larger size during the moult process (Wenner, 1985). Mykles and Skinner (1985) propose that proecdysial muscle atrophy is the result of an increase in the breakdown of protein. An earlier study (Skinner, 1966) found that the muscle may be reduced by as much as 40% of its original protein in the land crab *Geocarcinus lateralis*. Following ecdysis the muscle is restored at a rate of amino acid incorporation five times the anecdyial rate (Yamaoka & Skinner, 1974).

Clearly crustaceans are well adapted for quickly gaining and losing muscle mass during moulting. It is therefore surprising that krill are one of only two groups – the other being *Daphnia* – reported to make use of this physiological process for the purpose of shrinking during adverse conditions (Green, 1956; Thomas & Ikeda, 1987). Shrinking has been observed in laboratory maintained krill (e.g. Ikeda & Dixon, 1982; Nicol *et al.*, 1992; Sun *et al.* 1995) and appears to be common to all euphausiids studied so far. This has led researchers to propose shrinking as an over-wintering strategy for krill. It has been supposed that krill encounter low food availability in winter, where in fact krill have been shown to

demonstrate considerable flexibility in their diet and apparently do not starve during winter. As discussed in the section above on food availability and growth, krill may only infrequently encounter food shortages significant enough to cause shrinkage. This has led to some speculation that krill may not actually shrink under natural conditions (Siegel & Nicol, 2000).

The increasing use of modern, conservative techniques of measuring krill growth, (discussed in the next section) has provided a body of data which has been used to more confidently estimate growth rates in the field (Kawaguchi *et al.*, 2006). Analysis has shown small or negative growth across all length classes by autumn, confirming that shrinkage does occur in krill under natural conditions. Subsequent modeling of krill growth (Candy & Kawaguchi, 2006) found that incorporating shrinkage into the growth trajectory model gave more realistic outcomes for final krill size, for both the Indian and Atlantic sectors of the Southern Ocean.

Shrinkage is accompanied by a regression of sexual characteristics (Thomas & Ikeda, 1987), thus an apparently juvenile krill is not necessarily a young krill. Intensive examination of shrunken animals has found some differences attributable to shrinkage (Sun *et al.*, 1995; Shin & Nicol, 2002; McGaffin *et al.*, 2002). These techniques are labour intensive, and not easily or widely applied to wild-caught animals. Shrinkage and sexual regression further complicate attempts to describe growth and ageing in this most plastic of organisms.

### *Measuring growth*

Understanding of krill growth has been greatly enhanced by an increase in experimental studies since the 1970s (Nicol, 2000). An expansion of research associated with the BIOMASS (Biological Investigations of Marine Antarctic Systems and Stocks) program was largely geared to estimating krill biomass as the fishery expanded (El-Sayed, 1994). It quickly became clear from these studies, however, that early assumptions about krill life history established from the *Discovery* investigations some 40 years earlier, may be incorrect. Nicol (2000) and Siegel & Nicol (2000) provide thorough reviews of the development of methods

used to estimate growth, and only very recent developments, and those that are directly relevant to this thesis will be discussed.

A system to measure natural growth in wild krill was developed with the advent of the Instantaneous Growth Rate (IGR) method. The technique uses the difference in size of parts of the krill (uropods and telson) at moult and its exuviae (Quetin & Ross, 1991). The increment of growth at moult and the frequency with which the krill moults determine growth rate (Ross *et al.*, 2000). This integrates growth under natural environmental conditions that the krill has experienced during the previous moult cycle. Typically data are only collected for a few days once krill have been captured, to minimize the effects on growth of being held in an aquarium system. Indeed, Kawaguchi *et al.* (2006) found that aquarium effects were discernable in growth rates as early as the second day of experiments.

Other methods of measuring growth rates in the field require repeated sampling of a population. This can be done by tracking a single swarm as in studies by Kanda *et al.* (1982) and Clarke & Morris (1983), (interestingly, these two studies have given widely disparate growth estimates of  $0.063 \text{ mm day}^{-1}$  and  $0.33 \text{ mm day}^{-1}$ ). More commonly, field growth rates are estimated by performing analysis of sequential length frequency distributions (LFD) on catches taken throughout a season (see Nicol, 2000; Siegel & Nicol, 2000). Studies using the latter approach have used different methods to separate size classes. This will be discussed further in the following section.

The growth rates of krill have also been estimated from repeated hydro-acoustic surveys (Miller & Hampton, 1989; El-Sayed 1994), quantifying DNA:RNA ratios (Shin *et al.*, 2003), monitoring the change in size of krill in the diet of predators (Reid, 2001) and rearing populations in the laboratory (Murano, 1979; Poleck & Denys, 1982; Ikeda *et al.*, 1985; Ikeda & Thomas, 1987; Buchholz, 1991). Radiochemical labelling techniques (Roff *et al.*, 1994) have been used to quantify growth rates for other aquatic Crustacea, but have yet to be applied to krill.

Measured growth rates fall between  $+0.017$  and  $+0.163 \text{ mm d}^{-1}$  for larvae (Ross & Quetin, 1991; Huntley & Brinton, 1991),  $+0.047$  and  $+0.148 \text{ mm d}^{-1}$  for juveniles (Rosenberg *et al.*, 1986; Ikeda & Thomas, 1987), and  $-0.812$  and  $+0.37 \text{ mm d}^{-1}$  for adult krill (Quetin & Ross, 1991; Hewitt & Demer, 1994). These reported values come from various seasons, different size krill within each group

and often gender is not specified. Kawaguchi *et al.* (2006), however, report sex related differences: the growth rate for 40 mm female krill in January and February was 0.063-0.050 mm d<sup>-1</sup> but was significantly higher for males of the same length at 0.119-0.090 for the same period. Growth rates estimated from composite LFDs which do not take into account gender may therefore contain inaccuracies (Kawaguchi *et al.*, 2006). Growth rate estimates are therefore fairly wide and subject to some uncertainty, particularly as many of these estimates come from laboratory rather than field experiments.

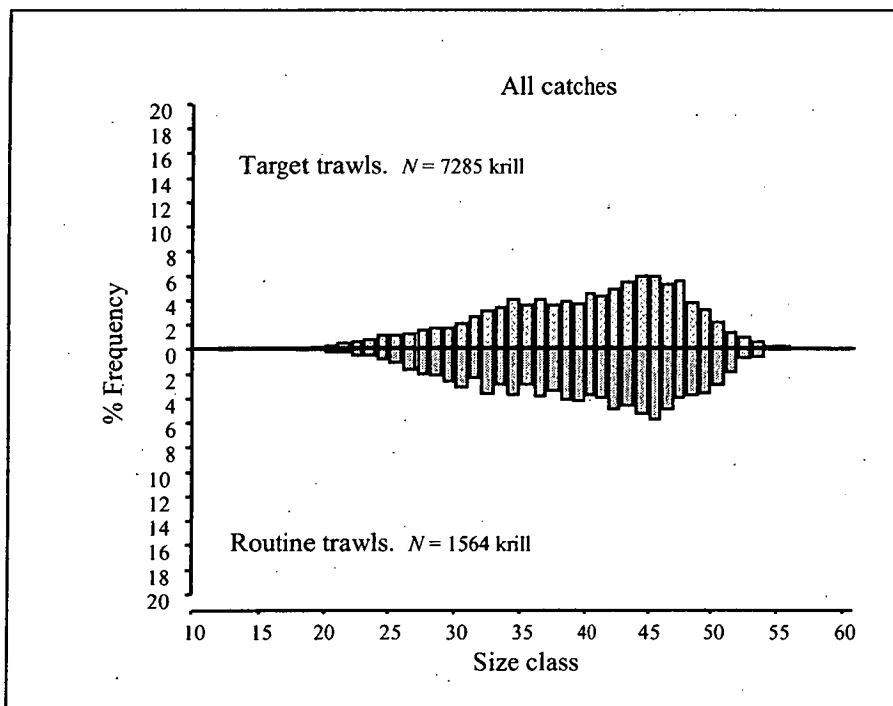
### *Ageing*

Krill have defied attempts to age them using conventional techniques; they lose their only hard part – the exoskeleton – at each moult, discarding any historical record of growth, such as growth rings in otoliths, shells, scales or fins that other marine organisms might retain. The evidence that krill have the capability of shrinking under adverse conditions, concurrently losing sexual characteristics (Ikeda & Dixon, 1982), means that size and maturity are not clearly related to age.

The comprehensive *Discovery* cruises of the 1930s laid the foundations for research on krill demography. Marr (1962) in particular, made significant observations about the life history of krill (e.g. Miller & Hampton, 1989; Nicol, 2000). The subsequent *Discovery* reports developed a life history model for krill based on these early investigations (Fraser, 1936; Bargmann, 1945; Marr, 1962; Mackintosh, 1972), which described krill as a short lived species that matured, spawned and died within 2-3 years. The predominant basis for this theory was the observation of two distinct modes in the measured length of captured post-larval krill. One clue that a 2-3 year life-span was a false conclusion was the relative abundance of the different size classes. In any population there must be fewer larger, older animals than there are small, young ones, due to mortality, but from length-frequency distributions (LFDs) this did not appear to be the case for krill (Figure 3). If krill reach close to their maximum size in their second year and live longer than 2-3 years, the observed LFD occurs because there is a piling up of cohorts in one larger size class (Ettershank, 1983a). The large/older cohort must be many year classes that strongly overlap and are not easily separated. Analysis of

LFDs therefore gave contradictory results because it required a decision about where to separate cohorts (e.g. Ivanov, 1970; Aseev, 1983 cited in Siegel, 1987).

Using size composition analysis to separate age classes and estimate longevity has remained a standard technique, but estimates of life-span have gradually increased over the years as other biological characteristics have been investigated. Observations of multiple spawning (e.g. Makarov 1975; Fevolden, 1979), growth and maturation rates (Poleck & Denys, 1982), and laboratory rearing (Mauchline, 1980) suggested a life-span of at least 3-4 years.



**Figure 3.** Length-frequency distribution of Antarctic krill captured off East Antarctica (80-150°E) during January-March 1996. (Australian Antarctic Division data).

More recently statistical analysis programs such as ELEFAN (electronic length frequency analysis; Pauly & David, 1981), Macdonald & Pitcher technique (Macdonald & Pitcher, 1979), or MIX analysis (Software for Mixture Distributions, Macdonald & Green, 1985) have been used in demographic studies to separate strongly overlapping size-classes in LFDs to estimate growth and age, giving life-span estimates of 6-7 years (Rosenberg *et al.*, 1986; Siegel, 1987; McClatchie *et al.*, 1991; Pakhomov, 1995a). This increased estimate of longevity has been supported by improved laboratory rearing studies (Ikeda, 1985; Ikeda *et*

*al.*, 1985; Ikeda & Thomas, 1987) and age-pigment analysis (Ettershank, 1983b, 1984, 1985; Berman *et al.*, 1989).

‘Age-pigments’ are the fluorescent lipid-based products of cellular degeneration. They accumulate within some post-mitotic cells over the life of an organism, and can therefore provide a proxy for age when the rate of accumulation is known. Age-pigment analysis has been the focus of much recent interest and will be discussed in depth in Chapter 2.

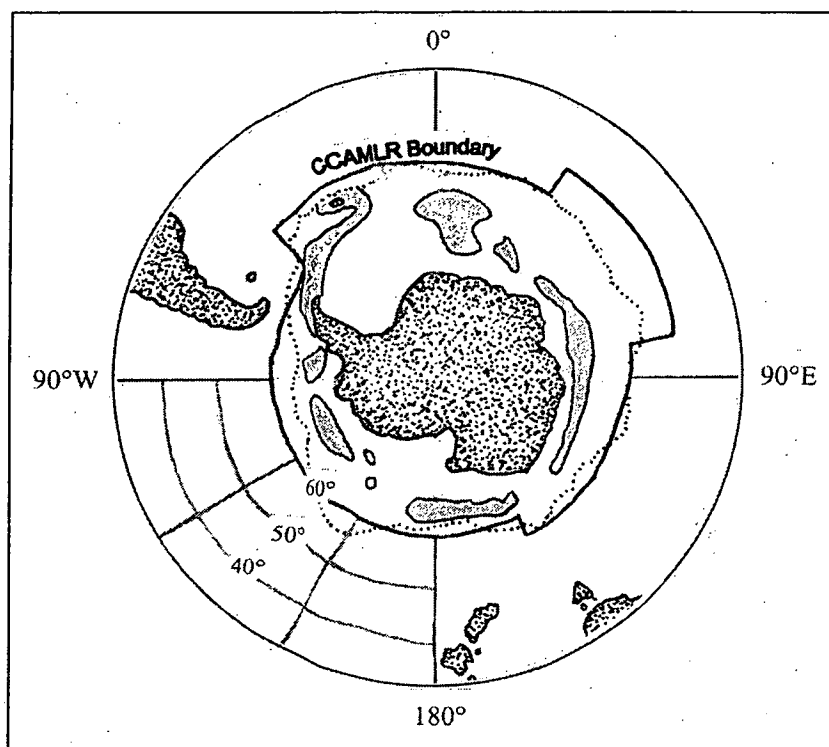
## **1.5 The krill fishery and its management**

Commercial fishing for Antarctic krill is undertaken by several nations. Historically Japan and the former U.S.S.R. dominated the operation of commercial krill fisheries in the Antarctic. Norway, Japan, Poland, the Republic of Korea and the Ukraine are the major nations currently fishing for krill (Nicol, 2008). Harvesting reached its peak in 1981/82 at 530 000 tonnes, (Nicol & Endo, 1999). The krill fishery has since been stable at a much lower level, most likely due to processing problems and a lack of demand. Harvesting remains much lower than the resource can potentially support (Nicol & Endo, 1999) at around 120 000 tonnes/year (Nicol, 2008).

Fishing has occurred in the South Indian, South Pacific and South Atlantic oceans. At present krill are only taken commercially in the Atlantic sector of the Southern Ocean (Nicol, 2008), particularly around subantarctic islands. The region from which krill are harvested may increase and become more circumpolar if the fishery expands.

A regulatory body was set up in 1981 to manage the harvesting of Antarctic marine living resources, of which krill is of particular interest because of its high conservation value. The Commission for the Conservation of Marine Living Resources (CCAMLR) manages krill over much of its natural distribution (Figure 4). Conservative or “precautionary” catch limits are currently set by CCAMLR to minimize the risk of over-fishing in the presence of incomplete biological information and a fluctuating environment. A krill yield model (KYM) was developed from the analysis of demographic information about krill (CCAMLR, 2006), which requires an estimate of biomass, and a factor which summarises the variability of life history characteristics such as growth and

mortality in krill. The generalised yield model (GYM) is a development of the KYM that is currently used to estimate annual yield of krill stocks (Constable & de la Mare, 1996). As with any fisheries model of this nature, decision rules are applied to limit the likelihood of over-exploiting the resource and give a 'sustainable' catch limit.



**Figure 4.** Boundary of the CCAMLR management area relative to krill distribution (shaded areas). The dotted line indicates the relative position of the Antarctic Convergence. Redrawn from Miller & Hampton (1989); Everson (2000) and Miller & Agnew (2000).

The growth model used in the GYM incorporates the 'trajectory of total length with age' (Candy & Kawaguchi, 2006) based on the von Bertalanffy (VB) model (Rosenberg *et al.*, 1986; Siegel, 1987). VB models are based on identifying cohorts from LFDs of population samples. As already discussed, age is not clearly related to size, so there are some obvious problems associated with constructing the growth trajectory using this method. Using an alternative source of growth estimation, such as the IGR method, appears to be a much more reliable basis for construction of growth trajectories (Candy & Kawaguchi, 2006).

There are two significant dangers that must be accounted for in the management process. Fluctuations occur in krill populations with environmental variability, and areas that are heavily targeted by fisheries are also those areas that

support large wildlife colonies. Setting a general catch limit over a wide area does not prevent the entire catch from being concentrated in a small part of that area, depleting krill stocks that are crucial to supporting wildlife hot-spots. In a 'poor krill year' - a year when the krill population has decreased - concentrated fishing effort that would normally be sustainable could have a significant effect on krill predators. CCAMLR is addressing this through the development of small scale management units (SSMUs) to reduce overlap between krill predators and the fishing industry (CCAMLR, 2006).

CCAMLR uses international cooperation, a large body of data and detailed analysis to achieve its goal of setting sustainable catch limits for the krill fishery. The short-fall in the management of the krill fishery lies, therefore, not with inadequate treatment, but with uncertainties regarding the biological inputs to the yield models. Estimates of biomass vary widely (for example see Everson & de la Mare, 1996; Siegel, 2005 and Atkinson *et al.*, 2008), the factors that cause fluctuations of krill biomass in an area are poorly understood (Nicol, 2003), and as discussed in section 1.4, growth, ageing and mortality remain poorly understood. In the interests of protecting krill as a species, and the Antarctic ecosystem, it is imperative that these biological characteristics are fully investigated and understood.

## **1.6 Aim of this research**

This research has been undertaken to provide improved information on krill growth and ageing. The primary aim of this thesis is to investigate and establish a reliable method of accurately ageing krill. During the course of this research other closely linked biological parameters that are integral to ageing will also be investigated, particularly growth and mortality. Previous research on ageing is discussed in Chapter 2 to provide background information, and to identify the most promising way to advance understanding of ageing in krill. The major goal is **to investigate the relationship between krill size, age and an appropriate biological age indicator** (chosen after extensively reviewing the available literature).



## **1.7 Research approach**

To achieve the aims and goals of this thesis, this chapter has provided necessary background information about Antarctic krill, the current state of knowledge on growth and ageing in krill, and a brief discussion regarding the CCAMLR model used for the management of the krill fishery. In Chapter 2, metabolism and the ageing process is explored. Knowledge about the biochemistry of ageing, and how this has been used to estimate the age of experimental animals, is discussed. How this might be applied to Antarctic krill is also considered. From this information, hypotheses about ageing are developed to be tested on krill. Both known-age reared krill and wild-caught krill are used in this study. The experimental methods that are used (those common to the whole study) are detailed in Chapter 3. The reasons for choosing these methods are given, and the design of the experiments is described.

Results are given in Chapters 4-6. In Chapter 4 the relationship between diet, temperature and mortality in captive krill is documented. Chapter 5 describes the relationship between known age, size, and the quantity of extractable fluorescent age-pigment (FAP) in the eyestalk ganglia. The effects of temperature, diet and stress on the accumulation of FAPs in krill (Chapter 6) and the growth of krill are also investigated.

In the general discussion (Chapter 7) the results of this study are compared with what might have been expected from existing theory, and new theories are developed. Testing of the hypotheses about the accumulation of FAPs with age, and how this might be used to age krill is also discussed. The findings of this study are examined with reference to population modeling, and how this work contributes to the current body of knowledge is considered. In the final chapter conclusions and recommendations are given.

## Chapter 2: Review of ageing theory & experimental studies

---

### 2.1 Introduction

In the previous chapter the requirement to determine the age of krill was outlined and the difficulty of ageing krill using conventional methods was discussed. There is, therefore, a need to investigate techniques that might be used directly to age krill, or which might be used as a proxy for age. To do this, there must be an understanding of both ageing theory and the biochemical and physiological mechanisms of ageing.

Laboratory experiments have shown that individual krill can be long-lived (up to 11 years) (e.g. Ikeda, 1985), and that the developmental processes continue well past the developmental stages of the first two to three years through to sexual maturity. Despite the pressures of predation, periodic starvation and infection, it is estimated that an individual krill can expect to live up to 5-8 years and reproduce in several seasons (Siegel, 2000; Nicol, 2000). As krill live for several years after reaching maturity they will experience ageing, which is the result of metabolic processes and is integrally linked with growth and environmental conditions. As krill age in a physiological sense, it is highly probable that they retain some internal evidence of metabolic history and hence physiological age. The aim of this chapter is to discuss current ageing theory and examine physiological ageing, for the purpose of identifying a method that might be useful for ageing krill.

In this chapter ageing theory is discussed as it applies to krill. This is followed by a summary of the biochemistry of ageing, with particular attention on free-radicals or reactive oxygen species (ROS) and anti-oxidative systems, and the subsequent formation of age-pigments. Chapter 2 also encompasses a review of research that uses physiological ageing ('wear-and-tear') as a proxy with which to determine the age of animals and particularly krill.

## 2.2 Ageing theory

Ageing is a complex process that is generally defined as losing physiological vigour with increasing time since birth. This usually involves decreasing fertility and increasing mortality, although not all species appear to age in this sense (Finch, 1990). Ageing is generally attributed to 'biological wear-and-tear', although this explanation only goes part of the way to explaining senescence (Kirkwood & Austad, 2000). Several theories exist about the origins and mechanisms of ageing. In animals such as Antarctic krill, ageing may accelerate generational turnover, which would allow the species to adapt quickly to a changing or variable environment. It is unlikely, however, that old-age is a significant cause of mortality for wild animals such as krill, which are subject to strong forces of predation, starvation and disease (Kirkwood & Austad, 2000).

Ageing is probably a life-history trade-off for krill, involving a choice between allocating resources to somatic maintenance versus reproduction. Known as the 'disposable soma theory' (Kirkwood, 1977), somatic maintenance - which includes antioxidant systems and DNA repair mechanisms - 'is required only to keep the organism in sound physiological condition for as long as it has reasonable chance of survival' (Kirkwood & Austad, 2000) and presumably a reasonable chance of reproducing. This may be especially so with an iteroparous species such as krill, a species that is capable of reproducing in several seasons (Ross & Quetin, 2000; Quetin & Ross, 2001). Reproducing in multiple years allows for years of low recruitment (due to a variable environment) which would otherwise see the population decline markedly (Quetin & Ross, 2003). According to the disposable soma theory, krill should therefore invest a reasonable amount of energy into metabolic costs associated with somatic maintenance, as well as reproductive events. The theory predicts that the amount of energy devoted to maintenance is related to longevity, and in fact krill are relatively long-lived (Quetin & Ross, 2003).

Krill live in a highly variable environment, and exhibit a degree of plasticity that allows them to respond to a range of different conditions. Food and temperature are two particularly important factors influencing growth and longevity. Exposure to low temperatures and periods of starvation in winter, despite seeming counter-intuitive, may explain the longevity of krill to some

extent. Krill appear to be well adapted to periodic food limitation by reverting to a non-reproductive stage, shrinking and reducing metabolic rate (see Siegel & Nicol, 2000).

The key to longevity appears to lie partly with metabolic rate. Animals with low metabolic rates frequently have longer life spans, an observation that is the basis of the 'rate-of-living' hypothesis (Finkel & Holbrook, 2000). The reason why metabolism should determine longevity remained unclear until Harman (1957) proposed the 'free-radical' theory of ageing, which is popularly understood today. Harman postulated that endogenous oxygen radicals - a by-product of breathing air - caused cellular damage that could accumulate with time. The rate-of-living and the free-radical theories are now considered to be complimentary aspects of one process; with increasing metabolism there is an increase in oxygen intake and the subsequent generation of ROS (free-radicals), which determine the rate of accumulation of cellular damage (ageing) and hence longevity (Finkel & Holbrook, 2000).

Ageing is a complex physiological process that cannot be simply attributed to a single cause or fully described by one theory. For the purpose of this study, however, 'rate-of-living' and free radical damage are central to these investigations, and present a direction for this research. Metabolic rate is therefore primarily implicated in the (measurable) ageing process, and for that reason, a brief discussion of metabolism in relation to life history is relevant.

## 2.3 Metabolism and ageing

The metabolic rate of Antarctic krill has been measured in many experiments, giving a comprehensive range for body size, sex, water temperature, season and most recently with active swimming<sup>1</sup> (Table 1). As with other marine invertebrates, the metabolic rate is usually considered to be specified by the rate of weight-specific oxygen consumption. This discounts any significant anaerobic metabolism occurring, but this is probably a reasonable assumption for krill (see Miller & Hampton, 1989). Routine oxygen consumption rates fall between the limits of 0.4 and 1.2  $\mu\text{l O}_2 \text{ mg}^{-1} \text{ Dry Mass (DM) h}^{-1}$  for small krill (furcilia III

---

<sup>1</sup> In all but the last case, the natural movement of the krill was restricted.

| Dry Mass (mg) | Sex/ maturity | T (°C)/ season | Oxygen consumption indiv. ( $\mu\text{l O}_2 \text{ h}^{-1}$ ) | Metabolic rate ( $\mu\text{l O}_2 \text{ mg}^{-1} \text{ DM h}^{-1}$ ) | Reference                                                                              |
|---------------|---------------|----------------|----------------------------------------------------------------|------------------------------------------------------------------------|----------------------------------------------------------------------------------------|
| 50            | Adult         | 1.0            | 90-400                                                         | 1.8 – 8.0                                                              | Swadling <i>et al.</i> (2005)<br>Swimming at current speeds of 3-17 $\text{cm s}^{-1}$ |
| 0.4           | III larvae    | -1.0 (A)       | 0.16-0.48                                                      | 0.4-1.2<br>(mean 0.8)                                                  | Meyer <i>et al.</i> (2002)                                                             |
| 44            | Juv           | -1.0 (A)       | 18.0                                                           | 0.409                                                                  | Atkinson <i>et al.</i> (2002)                                                          |
| 148           | Adult         | -1.0 (A)       | 39.1                                                           | 0.264                                                                  |                                                                                        |
| 0.8           |               | 0.5 (W)        | 0.46                                                           | 0.578                                                                  | Torres <i>et al.</i> (1994)                                                            |
| 1.5           |               | W              | 0.75                                                           | 0.502                                                                  |                                                                                        |
| 5.8           |               | W-S-A          | 4.85 – 4.89 – 4.21                                             | 0.836 - 0.843 - 0.725                                                  |                                                                                        |
| 14.5          |               | W-A            | 5.08 – 7.50                                                    | 0.350 - 0.517                                                          |                                                                                        |
| 89.6          |               | W-S-A          | 25.9 – 43.8 – 61.7                                             | 0.289 - 0.489 - 0.689                                                  |                                                                                        |
| 175           |               | W-S-A          | 54.3 – 90.8 – 99.6                                             | 0.310 - 0.519 - 0.569                                                  |                                                                                        |
| 280           |               | W-S            | 64.1 – 179.2                                                   | 0.229 - 0.640                                                          |                                                                                        |
| 70            |               | -1.8           | 45.5                                                           | 0.650                                                                  | Opalinski (1991)                                                                       |
| 40            |               | -0.7           | 27.6                                                           | 0.690                                                                  |                                                                                        |
| 152           |               | -0.5           | 110.2                                                          | 0.725                                                                  |                                                                                        |
| 94            |               | 0.0            | 70.0                                                           | 0.745                                                                  |                                                                                        |
| 45            |               | 1.5            | 30.6                                                           | 0.680                                                                  |                                                                                        |
| 99            |               | -1.8           | 31.1                                                           | 0.314                                                                  | Rakusa-Suszczewski (1990)                                                              |
| 106           |               | -1.0           | 45.9                                                           | 0.433                                                                  |                                                                                        |
| 83            |               | 0.0            | 33.2                                                           | 0.400                                                                  |                                                                                        |
| 18            |               |                | 8.0                                                            | 0.445                                                                  | Ishii <i>et al.</i> (1987)                                                             |
| 39            |               |                | 15.9                                                           | 0.408                                                                  |                                                                                        |
| 95            |               | 0.2 – 5.0      | 42.0                                                           | 0.442                                                                  |                                                                                        |
| 214           |               |                | 89.7                                                           | 0.419                                                                  |                                                                                        |
| 275           |               |                | 93.8                                                           | 0.341                                                                  |                                                                                        |
| 47            |               |                | 26.4                                                           | 0.562                                                                  | Ikeda & Bruce (1986)                                                                   |
| 76            |               | -1.6 –         | 37.3                                                           | 0.491                                                                  |                                                                                        |
| 135           |               | -1.8           | 68.9                                                           | 0.510                                                                  |                                                                                        |
| 243           |               |                | 119.1                                                          | 0.490                                                                  |                                                                                        |
| 351           |               |                | 178.0                                                          | 0.507                                                                  |                                                                                        |
| 27            |               |                | 13.1                                                           | 0.486                                                                  | Ikeda & Mitchell (1982)                                                                |
| 77            |               |                | 33.8                                                           | 0.439                                                                  |                                                                                        |
| 143           |               | -1.0 –         | 58.2                                                           | 0.407                                                                  |                                                                                        |
| 238           |               | -1.1           | 90.7                                                           | 0.381                                                                  |                                                                                        |
| 354           |               |                | 123.5                                                          | 0.349                                                                  |                                                                                        |
| 100           |               | 1.0            | 63                                                             | 0.630                                                                  | Hirche (1983)                                                                          |
| 34            |               | -1.5           | 17.3                                                           | 0.508                                                                  | Segawa <i>et al.</i> (1982)                                                            |
| 171           |               |                | 60.2                                                           | 0.352                                                                  |                                                                                        |
| 38            |               | 0.0            | 21.6                                                           | 0.568                                                                  |                                                                                        |
| 245           |               |                | 147.7 - 208.0                                                  | 0.603-0.849                                                            |                                                                                        |
| 26            |               | 3.5            | 17.2                                                           | 0.663                                                                  |                                                                                        |
| 207           |               |                | 132.1                                                          | 0.638                                                                  |                                                                                        |
| 36            |               | 5.0            | 32.9                                                           | 0.915                                                                  |                                                                                        |
| 275           |               |                | 233.5                                                          | 0.849                                                                  |                                                                                        |
| 40            |               | -0.5           | 15.3                                                           | 0.382                                                                  | Ikeda & Hing Fay (1981)                                                                |
| 25            |               | -1.0           | 17.1                                                           | 0.685                                                                  | Kils (1979, 1981)                                                                      |
| 70.5          | Juv           |                | 29.9                                                           | 0.424                                                                  | Rakusa-Suszczewski & Opalinski (1978)                                                  |
| 202.7         | M             | -1.0           | 84.5                                                           | 0.417                                                                  |                                                                                        |
| 141.7         | F             |                | 60.4                                                           | 0.426                                                                  |                                                                                        |
| 57.2          | Juv           |                | 40.0                                                           | 0.700                                                                  |                                                                                        |
| 249.5         | M             | +1.5           | 90.6                                                           | 0.363                                                                  |                                                                                        |
| 213.6         | F             |                | 109.8                                                          | 0.514                                                                  |                                                                                        |
| 29.4          | Juv           |                | 28.0                                                           | 0.954                                                                  |                                                                                        |
| 103.5         | M             | +2.4           | 36.2                                                           | 0.350                                                                  |                                                                                        |
| 93.7          | F             |                | 37.2                                                           | 0.397                                                                  |                                                                                        |
| 120           |               | 5.0            | 54                                                             | 0.450                                                                  | Chekunova & Rynkova (1974)                                                             |
| 45            |               | 0.0            | 31.0                                                           | 0.690                                                                  | McWhinnie & Marciniak (1964)                                                           |

**Table 1.** *Euphausia superba*: experimentally determined metabolic rates of krill. Wet weights given in Torres *et al.* (1994) were converted to dry mass using the relationship given by Ju & Harvey (2004; Figure 1). W=winter, S=spring, A=autumn. See references for more information on respirometer volumes, number of krill, acclimation and incubation times.

larvae<sup>2</sup>) in autumn (Meyer *et al.*, 2002), and have been measured as low as 0.2  $\mu\text{l O}_2 \text{ mg}^{-1} \text{ DM h}^{-1}$  for large (1g+) krill in winter, (Torres *et al.*, 1994; see Table 1). With the exception of the first entry (Swadling *et al.*, 2005), the data given below are for krill that are not actively engaged in swimming and are therefore roughly comparable (Table 1).

The example of the range of oxygen consumption rates given above summarises the overall pattern observed in metabolic rates for krill. Firstly that weight-specific metabolism generally decreases with increasing body size, (in fact Clarke and Morris (1983) stated that as body mass doubles, oxygen consumption per gram DM decreases by 4-30%). Secondly, metabolism appears to be significantly reduced in cold conditions and particularly in winter. Winter metabolism has been reported to be reduced by as much as 33% and 45% of the spring and autumn rates (Kawaguchi *et al.*, 1986; Quetin & Ross, 1991; Torres *et al.*, 1994), which may contribute to the over-wintering strategy of krill when food is less abundant.

That body size is directly related to oxygen consumption (Figure 5a) implies that krill are subjected to less oxidative stress as they get older, which may be a key to 'somatic maintenance' in the later, reproductive years, and a contributing factor to a long life span. The relationship between oxygen consumption  $\dot{QO}_2$  and body weight  $W$  is given by the equation:

$$\dot{QO}_2 = aW^b \quad (1)$$

which is the general form of a power curve, where  $a$  is the intercept (the metabolic rate when body weight  $W = 1$ ) and  $b$  is the body weight exponent (Withers, 1992). The power curve can be transformed to a linear relationship by taking the  $\log_{10}$  of both metabolic rate and body weight values:

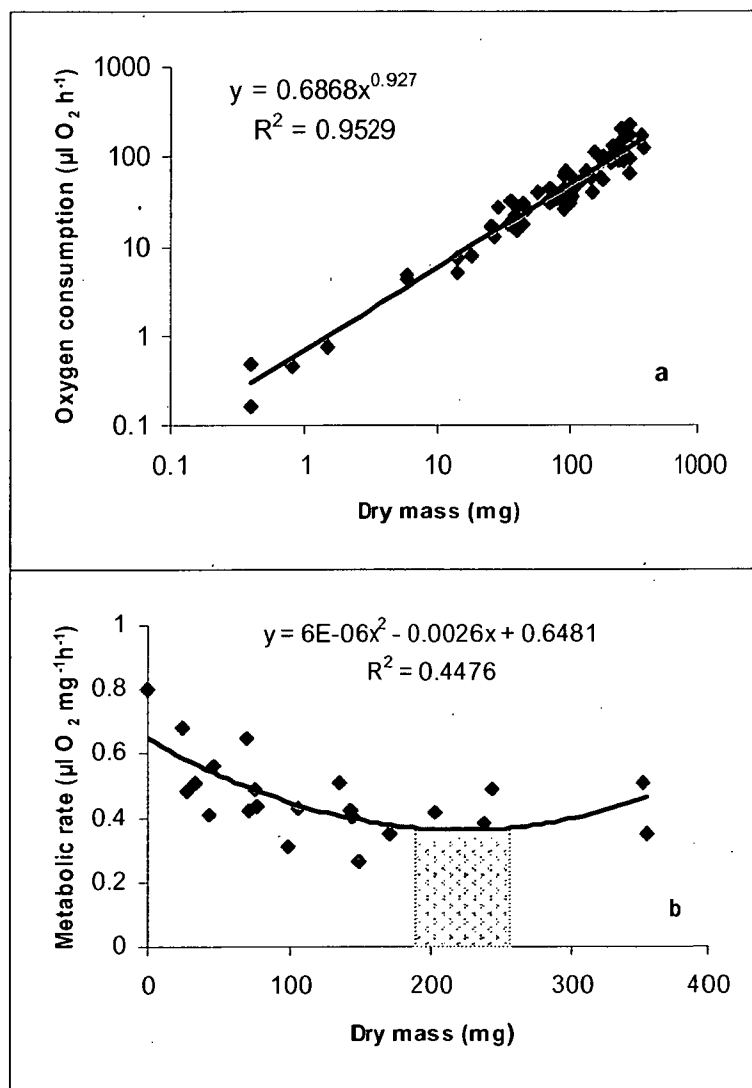
$$(\log_{10} \dot{QO}_2) = (\log_{10} a) + b(\log_{10} W). \quad (2)$$

as shown in a plot of the data given in Table 1 (Figure 5). The majority of values given by these authors represent a routine metabolic rate for krill, which

---

<sup>2</sup> See Kirkwood (1984) for an explanation of developmental stages.

presumably maintain pleopod beating to remain within the water column of the respirometer (Kils, 1981), but are not engaged in active feeding or swimming. The exception is Swadling *et al.* (2005) who measured oxygen consumption at various current speeds to assess the cost of swimming, finding much elevated  $QO_2$  values (and metabolic rates an order of magnitude higher, Table 1) compared with earlier research, and concluded that swimming could account for up to 73% of total daily metabolic expenditure.



**Figure 5.** Relationships between body weight and (a) oxygen consumption and (b) routine metabolism of Antarctic krill, *Euphausia superba*. Figures are constructed using data shown in Table 1, excluding Swadling *et al.* (2005) as their data are based on active swimming. Only mean values for oxygen consumption ( $QO_2$ ) and dry mass (DM) (as expressed in the original source publications) are plotted to illustrate the relationship;  $QO_2 = 0.6868 \text{ DM}^{0.927}$ . Temperature, seasonal and sex differences are ignored in (a) which explains most of the variance. Only data recorded for the temperature range  $-1.0$  to  $-1.8$  °C are used in (b). Metabolic rate is given as weight-specific oxygen consumption. The shaded area in (b) indicates the range of body size at which metabolic rate is apparently lowest.

It is very likely, therefore, that oxygen consumption rates are much higher for wild krill than these routine figures indicate, because for most of their lives they are obligate swimmers. It is useful, however, to compare routine metabolism data for a wide range of body sizes to look for patterns. Metabolic costs could also be expected to be related to sex, moult stage, over-all condition, gametogenesis, growth, feeding, digestion, and the proximity of other krill (Swadling *et al.*, 2005; Ritz *et al.*, 2002), however, the majority of studies have not taken these factors into consideration. Starvation has also been found to lower metabolic rate (Ikeda & Dixon, 1982; Atkinson *et al.*, 2002).

The relationship of decreasing metabolic rate with increasing body size described by Clarke and Morris (1983) only appears to explain part of what is occurring. By plotting all the published metabolic data available for Antarctic krill (Table 1) for one temperature class: -1 to -1.8°C (Figure 5b), it can be seen that the relationship is polynomial. Metabolic rate drops with increasing body mass through the larval, juvenile and small adult stages of development, but then rises again as mature adult krill get larger. This may be explained by the rapid somatic growth and hence high oxygen requirements of developing animals, which slows down as they near maturity, followed by a (seasonal) period of renewed development for reproduction.

Interestingly, there is a range of body sizes from 190-255 mg DM (0.76 – 1.02g wet mass) at which adult krill have the lowest metabolic rates (around 0.38  $\mu\text{l O}_2 \text{ mg}^{-1} \text{ h}^{-1}$ ). This may be crucial to understanding the lowered metabolic rates observed in krill over winter, which may not be caused simply by a reduction in available food, (i.e. krill don't need as much oxygen when not actively feeding and digesting) but are possibly related to shrinkage. According to the data shown in Figure 4, smaller adults use less oxygen (and hence age more slowly) than very large adults.

It would seem therefore that metabolism or the rate-of-living of krill is reduced as krill reach adulthood and in periods of low food availability, enabling krill to extend their lifespan and survive bad years to reproduce in subsequent years. That krill can lower their metabolic rates means that their rate of ageing is also slowed down. To this end, any method of quantifying metabolic age of krill needs to take into account sex, size/weight, sea-water temperature, ability to swim freely and feeding conditions.

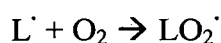
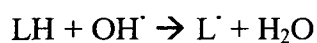


## 2.4 Oxidative stress and ageing

Ageing is a process that is determined by many factors, however, the key to longevity appears to lie with the generation of reactive oxygen species (ROS) and the body's response to this stress. Evidence such as the observation of increased lifespan in organisms with low metabolic rate, increased oxidative damage with age and identification of genes that are directly linked to ageing, strongly suggests that oxidants are related to ageing (Finkel & Holbrook, 2000; Camougrand & Rigoulet, 2001).

ROS include superoxide anions, hydroxyl radicals and hydrogen peroxide. Oxidants are generated as a result of normal metabolic processes, especially in mitochondrial electron transport chains, but may also be produced in response to external sources, such as exposure to ultra-violet radiation and environmental toxins (Finkel & Holbrook, 2000). The superoxide anion is not an aggressive oxidant whilst in aqueous solution; however it can be transformed to a hydroxyl radical in the presence of certain biological molecules such as hydrogen peroxide, with metal ions as catalysts in particular (McCord & Day, 1978). The hydroxyl radical is highly reactive and therefore can be very damaging to tissue.

Oxidative damage can occur to large bio-molecules including proteins, lipids and nucleic acids. Polyunsaturated fatty acids (PUFAs), which make up cell membranes, are particularly susceptible to oxidative damage because of their structure (Storey, 1996). This is principally because hydrogen atoms ( $H^+$ ) are readily removed from unsaturated lipid molecules leaving a carbon-centred radical lipid ( $L^{\cdot}$ ). The radical lipid reacts rapidly with oxygen ( $O_2$ ) to form the peroxyl radical ( $LO_2^{\cdot}$ ):



Lipid auto-oxidation, or peroxidation, proceeds by a chain reaction since the peroxyl radical can itself attack unsaturated lipids producing more radical lipid ( $L^{\cdot}$ ) and lipid hydroperoxide ( $LOOH$ ) (Storey, 1996). Lipid hydroperoxide breaks down to a suite of products including alkanes, alkenes, ketones and aldehydes. Malondialdehyde is perhaps the most important of these, as it can form Schiff

bases<sup>3</sup> with the amines of proteins, phospholipids and nucleic acids (Zielinski, 2000). The reaction end-products are large, auto-fluorescing molecules termed age-pigments, ceroid pigments and lipofuscins. These molecules are stored in lysosomes as cellular waste. In some post-mitotic tissues, such as neurons for example, this waste is not cleared and pigments accumulate over time, hence the name 'age' pigments (Leibovitz & Siegel, 1980).

Oxidative damage can be reduced by the action of anti-oxidants such as superoxide dismutase (SOD), catalase, glutathione and vitamins (Zielinski & Pörtlner, 2000). Anti-oxidant enzymes catalyse the breakdown of free-radicals, thereby minimising oxidative damage and are therefore important in reducing the rate of ageing. Diet is an important source of antioxidants, and must therefore be considered in any ageing physiology research.

## 2.5 Fluorescent age-pigments

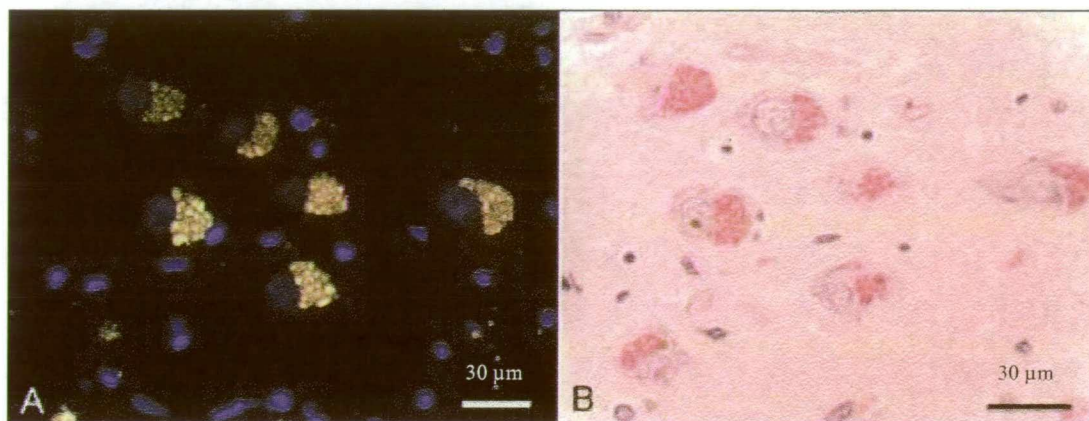
Pigmentary changes are an element of normal ageing, and in mammals appear externally as the greying of hair, the fading of eye colour and the formation of age spots and lipofuscin. Hair and eye colour fade because production of the pigment melanin decreases, however melanin is not a useful correlate with age throughout an organism's lifespan. Lipofuscins, on the other hand, accumulate in a time-dependant manner by the process described above, and is considered to be 'the most consistent and phylogenetically constant morphological change of ageing' (Porta, 2002). For this reason it is also called an age-pigment. The characteristics of lipofuscin include sudanophilia (an affinity for an oil-soluble or Sudan dye), acid fastness (resistance to de-colourisation by acids during staining procedures), and auto-fluorescence around excitation wavelength 440nm, emission wavelength 600nm – properties which allow for the substance to be identified and quantified (Figure 6).

The term *lipofuscin* has been used interchangeably with *ceroid pigment*, which is misleading (e.g. Terman & Brunk, 1998). Despite having similar characteristics such as auto-fluorescence, ceroid pigments and lipofuscin differ in their tissue

---

<sup>3</sup> Schiff bases are compounds derived by the chemical reaction (condensation) of aldehydes or ketones with aromatic amines, for example:  $\text{RNH}_2 + \text{R}'\text{CHO} \rightarrow \text{RN}:\text{CHR}' + \text{H}_2\text{O}$ .

distribution and accumulation rates (Porta, 2002). Ceroid pigments are related to disease pathology, whereas lipofuscin accumulation is the result of long-term cellular wear-and-tear. Both have been described as an age-pigment due to their common origin in oxidative damage and accumulation in older subjects. However, where lipofuscin accumulates in a predictable fashion over time, and therefore correlates with age much in the same way that annular rings in a tree do, ceroid pigment does not accumulate in a predictable way with time. Ceroid pigments are more likely to be found in older subjects because they are more likely to be suffering from disease (Porta, 2002).



**Figure 6.** Lipofuscin in the cytoplasm of human neuronal tissue under ultraviolet light (yellow, figure A) and transmitted light (red, figure B). In B the lipofuscin is stained red using the periodic acid-Schiff staining method. Image © Gray & Woulfe (2005).

Some researchers maintain that lipofuscins are a family of molecules, rather than one specific homogenous substance (see Brunk & Terman, 2002). Studies reporting properties such as accumulation rate and fluorescence spectra varying from one species to another, or even within a species, suggest that several compounds are involved (Eldred *et al.*, 1982). Debate continues in recently published literature (Sheehy, 2008 vs Harvey *et al.*, 2008) as to the use of the term lipofuscin to describe auto-fluorescent age-pigment that has been quantified using different techniques, and may in fact be different substances. The substance identified using *in situ* histological techniques and fluorescence microscopy (e.g. Sheehy *et al.*, 1998; Kodama *et al.*, 2006) may not in fact be the same substance as that being solvent-extracted and measured spectrally (e.g. Ju *et al.*, 1999). These researchers however, apply the same term ‘lipofuscin’ to the age-pigment in each

case. Conversely, Lehane & Mail (1985) identify similar fluorescent pigments, but call them pteridines. Both techniques show correlations between the quantity of the pigment examined and the age of the subjects. Perhaps some caution needs to be used in labeling the substance 'lipofuscin', despite a precedence in the literature for general use of the term, (see Brunk & Terman, 2002). Porta (2002) discusses the implications of the 'misconception' that lipofuscin can be solvent-extracted.

As the nomenclature is subject to debate, it is more prudent to use the general term fluorescent age-pigment (FAP) in this study. The characterisation of the FAP molecule(s), whilst in itself is important and interesting research, is outside the field of this thesis. One of the key aims of this review is to identify a technique with which to determine a relationship between pigment and age in krill. The name of the substance, and an understanding of its structure, is of secondary importance. Subsequently, caution will be exercised when comparing results from this study with other published data produced by different techniques.

## **2.6 Using fluorescent age-pigments to age crustaceans**

Various forms of FAP work have successfully given age results for humans (Wing *et al.*, 1978; Dayan *et al.*, 1988; Brunk & Terman, 2002), fish (Girven *et al.*, 1993), insects (Mail *et al.*, 1983; Lehane & Mail, 1985) and crustaceans (Sheehy 1990a, Ju *et al.*, 1999). It has been used less successfully, however, for some insects, mice and crustaceans (e.g. Sheehy & Roberts, 1991).

Various methods of quantifying FAPs in crustaceans are currently available. They generally follow two major approaches: histological sectioning and staining of neural tissue (Medina *et al.*, 2000), frequently accompanied by fluorescence or confocal microscopy and digital image analysis (Sheehy, 1990; Belchier *et al.*, 1994; Wahle *et al.*, 1996a; Vila *et al.*, 2000; Bluhm *et al.*, 2001), and solvent extraction of pigments and analysis of fluorescence (Ettershank, 1983b; Ettershank, 1984; Sheehy & Ettershank, 1988; Nicol *et al.*, 1991; Ju *et al.*, 1999; Ju *et al.*, 2001; Ju *et al.*, 2003). Each of these techniques has advantages and disadvantages. Histological techniques are precise but laborious, whereas extraction techniques are much more rapid and can be applied to many more samples, but are less specific. Amidst debate on the application of ageing methodologies, each method has attracted some support from researchers (e.g.

Sheehy, 2008; Harvey *et al.*, 2008). Much of the debate centres on the exact composition of the fluorescent molecule that is being measured; as mentioned above, it is likely that the pigment that is quantified *in situ* differs from that which is solvent-extracted.

The techniques for examining FAPs have been refined over the past 20 years. Following initial excitement about the possibilities of using pigments to discriminate between age classes in “difficult-to-age marine invertebrate” species, Nicol (1987) discusses the problematic nature of examining specimens that are subject to various preservation methods. The three major methods of preserving crustaceans (storage in ethanol, in formalin, or freezing), produce differing fluorescence independent of age-class (Nicol, 1987). For frozen samples, freezer temperature is also important, as fluorescence in samples continues to accumulate at temperatures above -20°C (Hill & Womersley, 1991). The artefact fluorescence of chemical preservation can therefore be taken into account by examining only samples that are frozen, preferably at ultra-low temperatures, (<-70°C), and comparing results only with other studies that have treated their samples likewise.

## **2.7 Studies of fluorescent age-pigments in krill**

Age-pigments present a possible solution to the difficulty of ageing krill. For this reason, age-pigment research has been applied to krill over the past 25 years. Much of this research occurred between 1983 and 1991, during which time 8 of the 10 published investigations occurred (Table 2). Since Ettershank’s pioneering work in 1983, many studies have attempted to identify and quantify age-pigments in krill, but with limited success.

There are several reasons why this ageing technique has not yet been particularly successful. Firstly, there are methodological problems. The histological approach that has given good results in other species (section 2.6 above) has either shown no age-pigment in krill (Sheehy, 1990b) or very small quantities (Bluhm *et al.*, 2001). The problem is probably not the absence of pigment, but the difficulty in locating it. Taking replicable histological sections through krill neural tissue is manifestly difficult. The practice of finding FAPs in thin-sections of very small tissue samples, where the orientation may vary, can be

inconsistent. Large sample sizes might negate this problem, but for such a labour intensive procedure this is impracticable.

The extraction technique, which initially appeared to produce good results, has also been subject to methodological problems. The fluorescent component of an extract (from the brain of a freshwater crayfish) in the lipid solvent chloroform-methanol was shown to bear no quantitative relationship to morphological lipofuscin revealed by the histological technique (Sheehy, 1996). The author of this study argues that the relationship of fluorescence with age attributed by earlier authors, was actually a function of either organ or body weight. Further, Sheehy (1996) asserts that there is no evidence that the fluorescent extract is from age-pigment. The extraction process has recently been modified as technology has matured (Ju *et al.*, 1999; Ju *et al.*, 2001; Ju *et al.*, 2003). This refined method offers the possibility of ageing krill, and is worthy of further investigation.

| Species                                | Method                                                                                                         | Major outcomes                                                                                                                       | Authors                         |
|----------------------------------------|----------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------|---------------------------------|
| <i>Euphausia superba</i>               | Extraction of pigments in chloroform-methanol, fluorescence measured by spectrofluorimeter                     | Up to 5 age classes in mature females                                                                                                | Ettershank, 1983a               |
| <i>E. superba</i>                      | As above                                                                                                       | 3 year groups in gravid females                                                                                                      | Ettershank, 1984a               |
| <i>E. superba</i>                      | As above                                                                                                       | Handbook for methodology based on two prior studies                                                                                  | Ettershank, 1984b               |
| <i>E. superba</i>                      | As above                                                                                                       | 5 year adult life span for mature males, 1 year class for juveniles                                                                  | Ettershank, 1985                |
| <i>Meganyctiphanes norvegica</i>       | As above                                                                                                       | Preservation process affects fluorescence                                                                                            | Nicol, 1987                     |
| <i>E. superba</i>                      | As above                                                                                                       | 6 year classes for adult krill                                                                                                       | Berman <i>et al.</i> , 1989     |
| <i>E. superba</i>                      | Histology plus fluorescence microscopy                                                                         | No age-pigment found                                                                                                                 | Sheehy, 1990b                   |
| <i>E. superba</i>                      | As for Ettershank 1984b, but with freeze-dried whole krill instead of dissected formaldehyde preserved samples | Increase in pigment over 1 year in shrinking krill                                                                                   | Nicol <i>et al.</i> , 1991      |
| <i>E. superba</i>                      | As for Sheehy, 1990b                                                                                           | Found single, small lipofuscin granules, <0.01 % area fraction                                                                       | Bluhm <i>et al.</i> , 2001      |
| <i>E. pacifica</i> & <i>E. superba</i> | Extraction in DCM-methanol, automated sampling and fluorescence measured by HPLC                               | Known-age calibration for reared <i>E. pacifica</i> . Higher quantities of pigment in wild <i>E. superba</i> than <i>E. pacifica</i> | Harvey <i>et al.</i> , in press |

**Table 2.** Summary of research into fluorescent age-pigment in krill.

Secondly, there has been no other ageing technique available with which to validate any of the age-pigment approaches for krill. In fish, for example, otoliths accumulate growth rings (Secor *et al.*, 1995), which can be used to validate other ageing methods. For krill though, the best age estimates have been derived from length-frequency analysis (Siegel & Nicol, 2000), which can be problematic (see section 1.4 above).

Finally, age-pigment research for krill stalled because the technique could not be calibrated. Calibration requires animals of known-age. It is only recently that Antarctic krill have been reared from eggs in the laboratory (Hirano & Matsuda, 2003; Hirano *et al.*, 2003), thus providing a source of known-age krill from which ageing studies might be undertaken.

## 2.8 Summary

Ageing theories predict that krill age in a physiological sense and experience 'wear-and-tear', as they may live for many years after reaching maturity. As obligate swimmers, Antarctic krill have an active lifestyle, and therefore a high metabolic rate. High metabolism equates to high oxygen intake, which results in the by-production of reactive oxygen species (ROS) (e.g. hydrogen peroxide) that damage cellular components. The products of the lipid peroxidation of cell membranes further react with damaged proteins to produce a suite of auto-fluorescent waste products, which accumulate in post-mitotic tissue over time. These fluorescent molecules are known by several synonyms, including the terms *age-pigment* and *lipofuscin*.

Over the past 25 years, two major techniques for quantifying age-pigment have been developed and refined: *in situ* histological approaches, and solvent extraction with spectral analysis. Recent advances in the latter technique allow for studies in which greater numbers of animals can be examined more rapidly than if the laborious histological approach was undertaken. Thus, whilst not as precise at predicting age, pigment extraction provides a tool with which to examine larger population groups for evidence of cohorts. This approach is satisfactory where the identification and characterisation of the pigment, which is available via the histological approach, is of less importance. It is applicable chiefly to age

determination in organisms that have defied being aged using conventional methods, but because of ecological or economic importance there is a strong requirement for some description of population parameters. For Antarctic krill, as a keystone species in the southern ocean ecosystem and the target of a commercial fishery, this is particularly relevant.

## 2.9 Conclusions & hypotheses

The development of the pigment extraction technique for demographic studies of the blue crab *Callinectes sapidus* (Ju *et al.*, 1999; 2001; 2003) presents a possible solution to ageing Antarctic krill. In the present study, the biochemical extraction technique is used and refined with a view to correlating the accumulation of age-pigment in krill neural tissue with time. Factors such as temperature, gender and diet differences that krill might encounter under natural conditions are taken into account.

Based on the information given in Chapter 1 and above, it is rational and logical to develop and test the following hypotheses:

1. Nutrition, temperature and stress have a direct effect on growth.
2. Nutrition, temperature and stress have a direct effect on metabolism and therefore lifespan.
3. Fluorescent age-pigments (FAPs) accumulate with time in Antarctic krill;
4. FAPs can be extracted from krill and measured;
5. Factors which affect growth rate and metabolism will affect the accumulation rate of FAPs, and
6. The amount of FAP extracted can be used to predict the age of krill.

Antarctic krill are a globally important species, and there is a pressing need for better information about growth and ageing. The current literature indicates a very promising field of research in fluorescent age-pigments. It is therefore appropriate to further investigate how factors such as food, temperature and stress affect growth, ageing and mortality in Antarctic krill; and importantly, to pursue a method of extracting and quantifying FAPs from krill with a view to better age-



class separation than conventional methods permit. The following chapter describes the methodology used, the reasons this approach was taken and the design of the experiments to test these hypotheses.

## Chapter 3: Materials and methods

---

### 3.1 Overview

This chapter describes the methodologies selected and developed to test the hypotheses stated at the end of Chapter 2. It also provides an outline of the overall experimental design used in this study. More explicit details of chapter-specific methodology can be found in each results chapter.

### 3.2 Selection of methods

Initially, this research was designed to encompass two approaches to quantifying age-pigment: the histological/microscopy approach and a biochemical extraction technique, both applied to the same krill. This dual approach might provide a way to verify the extraction technique, since there is debate about the comparability of results obtained using these two techniques (see Sheehy, 2008; Harvey *et al.*, 2008). Applying and comparing these techniques would be possible by using the ganglion from one eyestalk for histology and using the other eyestalk for biochemical extraction.

Ultra-thin (6µm) transverse sections through the eyestalk ganglion of krill over a range of sizes were examined, however no age-pigment was detected. The technique was laborious and awkward because of the tiny amounts of tissue examined, and the difficulty in getting consistent orientation of the sample in the wax block. This is consistent with previous investigation where age-pigment was reported as being difficult to detect in krill using this technique, (Sheehy, 1990; Bluhm, *et al.*, 2001). Consequently, the histological technique was not pursued further and the study concentrated on various aspects of the extraction technique.

A review of the recent literature on age-pigment detection in Crustacea (Chapter 2, section 2.6) provided a refined protocol for biochemical extraction of age-pigment from another crustacean, the Blue Crab *Callinectes sapidus* (Ju *et al.*, 1999; Ju *et al.*, 2001; Ju *et al.*, 2003). Correspondence with this research group

resulted in a clarification of the procedure, and with some small modifications, this was adopted as the single method used in the present study to quantify age-pigment from krill. The technique is described in detail in section 3.6 below.

The capture of Antarctic krill for this research, and their subsequent maintenance in the laboratory, followed well-established methods and required little further development. The modification of feeding, temperature and stress conditions occurred within this framework, and is described in detail in section 3.4 below.

### **3.3 Experimental design**

The experiments were designed to test the hypotheses which were developed from the background material in the first two chapters. To summarise the hypotheses from Chapter 2: nutrition, temperature and stress have a direct effect on growth, metabolism and therefore lifespan (hypotheses 1 & 2); fluorescent age-pigments (FAPs) accumulate with time (age) in Antarctic krill and can be extracted and measured (hypotheses 3 & 4); factors which affect growth rate and metabolism will affect the accumulation rate of FAPs (hypothesis 5); if extracted pigments correlate with age (Hypothesis 3) they can be used to predict the age of wild krill (hypothesis 6).

To test these hypotheses, there are three main components to this age-pigment research:

1. Wild Antarctic krill were captured and held in aquaria under 'maintenance' conditions (see following section 3.4) over a period of 1 year and sub-sampled at intervals to measure growth and to identify an increase in an extractable fluorescent pigment with time. (To test hypotheses 3 & 4).
2. Concurrently, krill from the same initial wild population were maintained under a range of conditions, including different temperature regimes (0°C or 3°C), diet (limited energy diet, phytoplankton monoculture or mixed saturating diet) and stress (handled or not

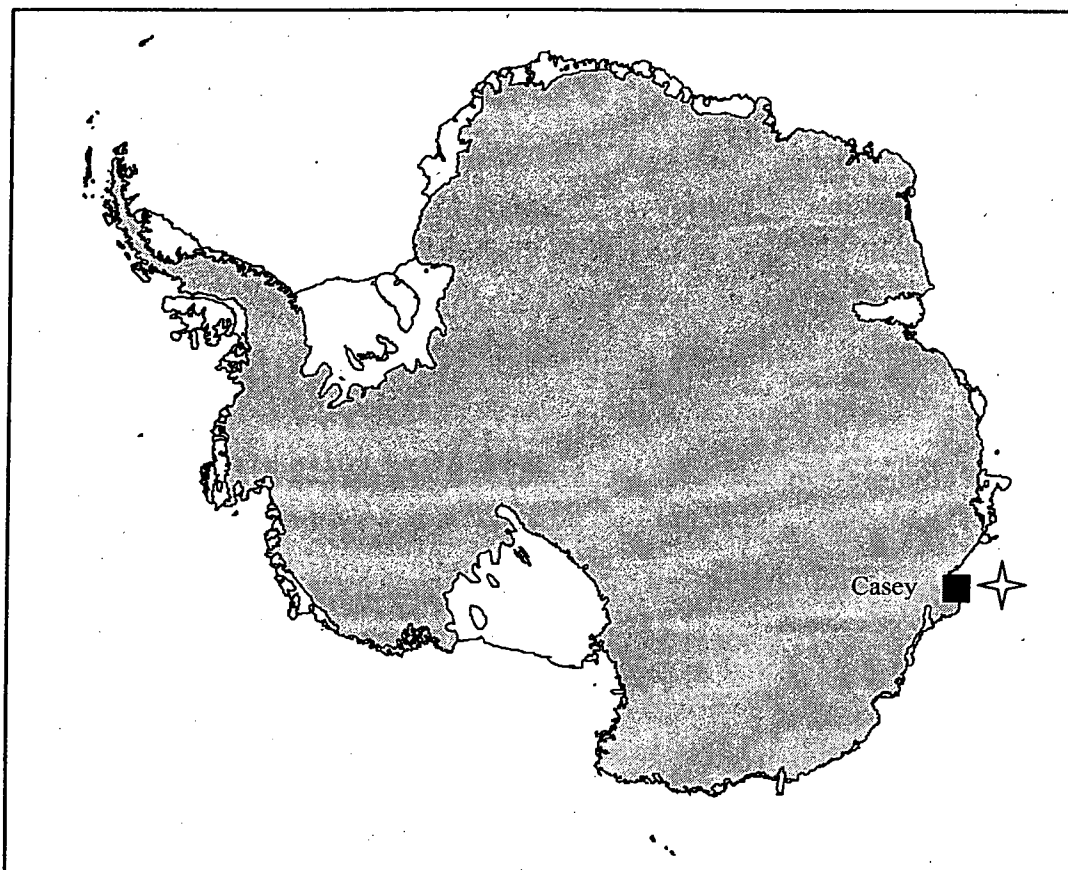
handled) to generate a range of metabolic responses. Growth, accumulation of pigment and mortality were examined. (To test hypotheses 1, 2, 3, 4 & 5).

3. Following the identification of a pigment that accumulates with time, known aged krill (raised from eggs in captivity) were sampled at four intervals over four years. Fluorescent pigments were extracted from post-mitotic neural tissue of these krill to identify and describe a relationship between pigment and age for both male and female animals (to test hypothesis 3). These data were used to develop a model to predict age in wild krill (to test hypothesis 6).

### **3.4 Krill collection & aquarium maintenance**

#### *Wild krill collection*

Krill used in this study were collected from 65° 8.2'S, 109° 42.15'E near Casey station (Figure 7) in Antarctic waters on 26<sup>th</sup> March 2001. The krill were collected using a fixed open Rectangular Mid-water Trawl (RMT) net towed for 10 minutes at a depth of 0-30 metres from the RSV *Aurora Australis*. A sub-sample of the catch was preserved for later analysis; 50 krill were placed individually in cryotubes and frozen in liquid nitrogen. The remaining live krill were placed in refrigerated flow-through sea-water tanks, and kept for one week during the return to Australia. Dead krill were removed from the tanks each day to maintain water quality. Upon arrival in Australia the krill were transferred to a 1000 litre aquarium at the Australian Antarctic Division (AAD) headquarters in Tasmania, Australia. The krill were kept in the aquarium in the dark at about 0°C for 1 month, and fed a live culture of the diatom *Phaeodactylum tricornutum* to allow the stress- and damage-induced mortality rate to subside.



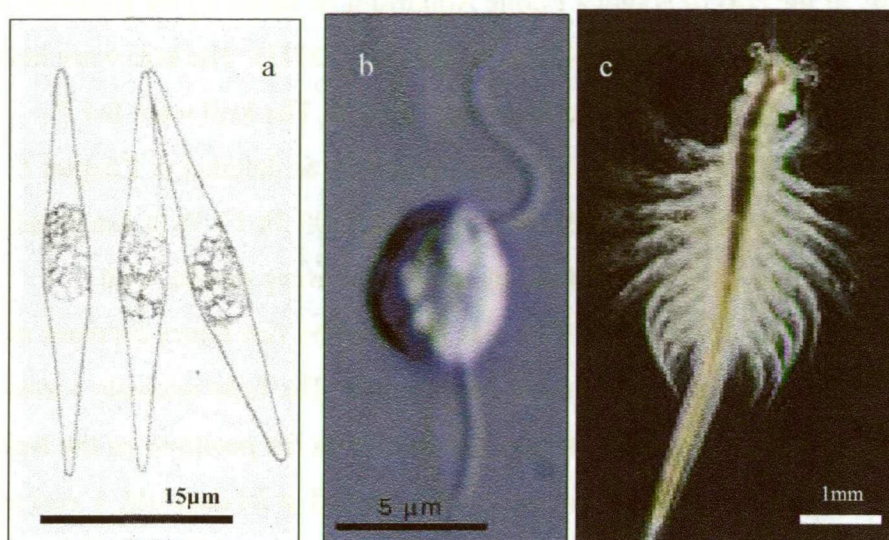
**Figure 7.** Map of Antarctica showing collection site of Antarctic krill *Euphausia superba* used in this study. The fishing position 65° 8.2'S, 109° 42.15'E is indicated by a star. Coastline data courtesy of the Australian Antarctic Data Centre © Commonwealth of Australia 2008.

### *Rearing conditions*

One month after the krill were captured they were moved into separate aquaria to begin the growth and ageing experiments. The krill were divided into 13 separate aquaria. The first aquarium was the previously mentioned 1000 litre aquarium, which held the majority of the animals. This was designated the 'stock' aquarium as it was the major reservoir of live krill available for experiments at the AAD. The number of krill in this tank was estimated to be approximately 3000.

600 krill were divided between twelve 20 litre aquaria at a stocking density of three krill per litre. Six of the 20 litre aquaria were kept at an ambient temperature of 0°C and six at 3°C. At each temperature, two aquaria received very low levels of the phytoplankton *Phaeodactylum tricornutum* (Figure 8a) (approximately 1.52E+04 cells ml<sup>-1</sup> once per week), two received saturating quantities of *P. tricornutum* (approximately 6.08E+04 cells ml<sup>-1</sup> per day), and two

received a saturating mixed diet, consisting of *P. tricornutum* ( $3.04 \times 10^4$  cells  $\text{ml}^{-1}$  per day) and a commercial aquaculture preparation of spray-dried *Schizochytrium* sp. (Figure 8b) marketed as 'Algamac 2000' ( $2.0 \text{ mg l}^{-1}$  per day). More details of the feeding regime are recorded in Chapter 4.



**Figure 8.** Food fed to krill in these experiments. Figure a: Three normal cells of the pennate diatom *Phaeodactylum tricornutum* Bohlin. The pair on the right is separating after division. Drawing © B. Wisely and C. Purday, 1963. Figure b: *Schizochytrium* sp. microalgae. Figure c: The brine shrimp *Artemia salina*.

The aquaria were monitored daily and the temperature recorded at approximately 24 hr intervals. The krill were fed, and 25%-50% of the water changed on a daily basis to remove nitrogenous waste and uneaten food, and to increase oxygen saturation. Any dead krill were removed from the aquaria and frozen in liquid nitrogen, then stored at  $-86^{\circ}\text{C}$  for later morphometric analysis. All exuviae were removed from the aquaria and discarded. Information on mortality, moult rate, and temperature was recorded.

At 30-day intervals the krill in one of each of the feeding regimes (three at each temperature) were carefully removed, and individually digitally photographed so that morphometric measurements could be made from the images. The remaining aquaria served as a control for stress as they were not handled for measuring. At 3-monthly intervals the krill in all aquaria were sub-sampled, and usually 10 (when available) from each group were frozen in liquid nitrogen for morphometric and biochemical (pigment) analyses. The experiments were run until all krill had been sampled or had died.

### *Known-age krill*

Known-age Antarctic krill were made available for this study by raising krill from eggs in an aquarium. Wild krill from the West Atlantic sector of the Southern Ocean and from AAD stock sourced from East Antarctica, were maintained in captivity at the Port of Nagoya Public Aquarium in Japan for the purpose of breeding (Hirano *et al*, 2003; Hirano & Matsuda, 2003). The krill were induced to spawn and the hatchlings raised from egg to adult. The krill were fed *P. tricornutum* and *Artemia salina* (Figure 7a and c), as detailed in Chapter 6. The photo-period was seasonally adjusted to give L:D=5.5h:17.5h in June, and the light period was progressively increased by two hours every month, with L:D=17.5h:5.5h in December. This was followed by two hours decrease of light period every month until reduced to June regime. The light intensity varied between 25-450 lux at water surface, depending on the positions of the tanks in relation to light source. The krill were sub-sampled at 30 days old, 1 year old, 3 years old and 4 years old. Individual live krill were carefully placed into a labelled cryotube and frozen in liquid nitrogen. The frozen krill were shipped to the AAD, where they were defrosted and subjected to morphometric analysis, dissection and pigment analysis.

### **3.5 Collection of morphometric data and image analysis**

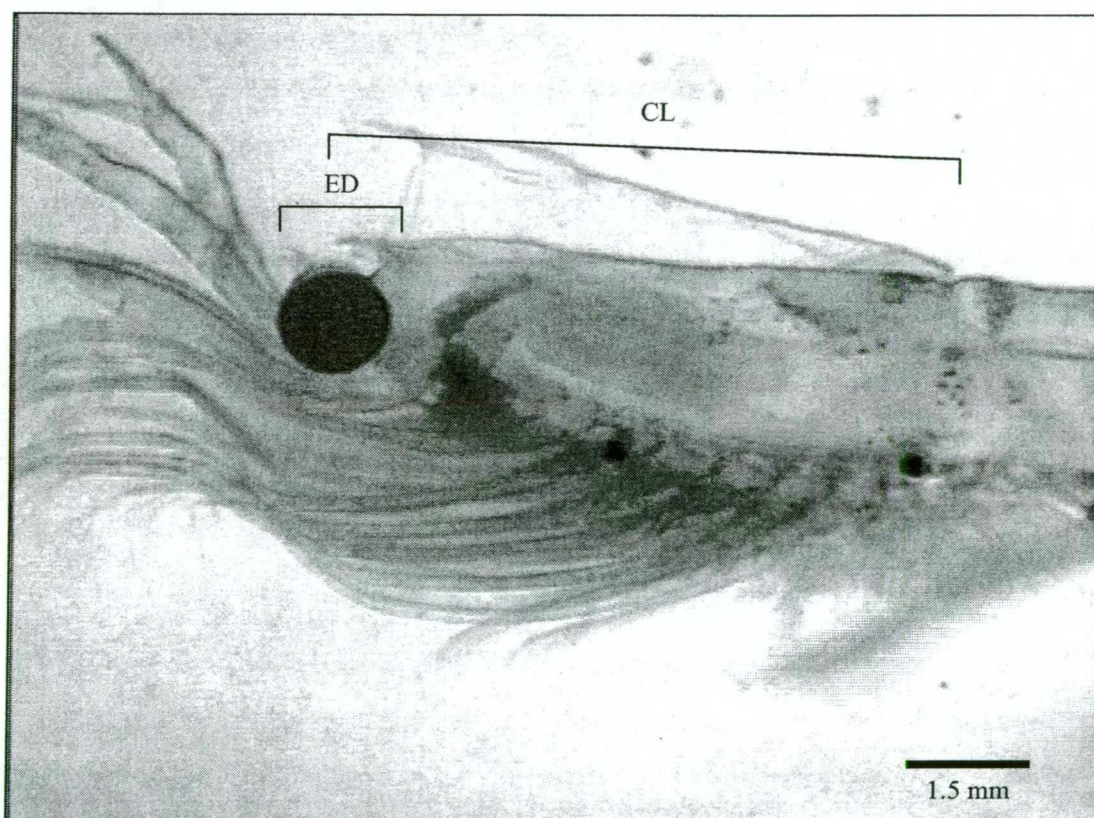
Morphometric data was collected from both frozen and live krill. Frozen krill were defrosted from  $-86^{\circ}\text{C}$  by placing individual cryotubes in a  $-20^{\circ}\text{C}$  freezer for 20 minutes, then transferring to an ice-water slurry for several minutes. Each krill was then removed from the cryotube by flushing with chilled sea water into a glass Petri dish. Live krill were transported between the aquaria and the microscope laboratory in 2 litre containers of seawater maintained at approximately  $0^{\circ}\text{C}$  by being packed in ice within an insulated cold box.

Krill were examined individually under a dissecting microscope using a cold light source. All krill were oriented on their right hand side and photographed digitally using a Sony video camera attached to the microscope, and the images saved to an IBM personal computer with an identification code. The carapace length and the eye diameter of the krill were measured on the digital image (Figure



9) using the image analysis program SigmaScan Pro v5 software (Jandel Corp.). The krill were sexed according to Kirkwood (1982).

The eyestalks were carefully excised from the defrosted krill, so that the eyestalks remained attached to each other at the base and the remainder of the body was refrozen in the original cryotube in liquid nitrogen, and returned to storage at -86°C. The eyestalks, including eyeballs, were momentarily blotted on paper towel and weighed on a micro-balance in a labelled, tared 2 ml amber glass HPLC vial.



**Figure 9.** Antarctic krill *Euphausia superba*. The morphometric measurements taken for eye diameter (ED) and carapace length (CL) are shown. This krill was alive when photographed and in the process of moulting.

### 3.6 Pigment extraction

Extraction was undertaken immediately to reduce the potential error due to handling or the preservation process, which have been shown to alter the fluorescent characteristics of the tissue (Nicol, 1991; Hill & Wormersley, 1991; Ju *et al.*, 1999). A published method was used (Ju *et al.*, 1999; Ju *et al.*, 2001; Ju *et*



*al.*, 2003), with some modifications as follows: bulk 2:1 v/v dichloromethane-methanol solvent was prepared by adding 200 ml of methanol to 400 ml dichloromethane (DCM) in a 1 litre bottle in a fume-hood. The bottle was capped with a 1ml dispenser. Then 1 ml of mixed solvent DCM-methanol (2:1 v/v) was added to the vials containing the excised eyestalks which were then capped. Teflon cap liners were inserted into the caps using clean forceps. These were used to prevent the solvent reacting with the plastic cap. The vials were placed in a bath sonicator (40W) for one minute to aid extraction of soluble pigments, and then centrifuged at 800g for 10 minutes at 4°C to remove cellular debris. Half of the extract (500 µl) was quantitatively transferred by micro-pipette to a glass vial insert<sup>4</sup> in an amber glass vial, and the pair of vials dried under liquid nitrogen. Dried samples were redissolved in 250 µl of methanol. The pair of vials were given different coloured caps to identify which contained the cellular debris and which the extracted pigment, and stored immediately in labelled vial boxes at -86°C until all samples were extracted in preparation for the fluorescence assay.

### 3.7 Measurement of fluorescence

#### *Equipment*

The fluorescence of the extracted pigments, sample protein and the calibration compounds was measured using an Agilent 1100 series HPLC (Figure 10). The HPLC comprised of the following components: degasser model G1322A, quaternary pump model G1311A, auto-sampler model G1313A and fluorescence detector model G1321. Analysis was undertaken at the Department of Primary Industries Weribee Laboratories (Victoria, Australia).

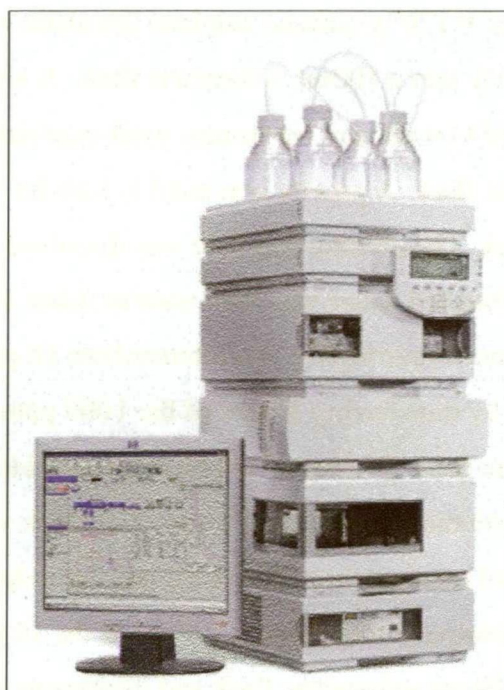
A sub-set of samples was analysed at a remote laboratory at the University of Maryland Center for Environmental Science (UMCES), where this technique was developed, also using an Agilent HPLC. This was done in the first instance to verify that the technique could detect measurable amount of pigment in Antarctic krill, and secondly to provide a comparison of results.

---

<sup>4</sup> A small glass tube inserted into an HPLC auto-sampler vial so that very small quantities of liquid can be taken-up and injected.

### *Pigment fluorescence*

Volumes of 20  $\mu$ l from each pigment extract were injected by auto-sampler (Agilent 1100 series HPLC) with methanol as the carrier solvent (0.8 ml min<sup>-1</sup>) through the flow cell (volume 16  $\mu$ l). The major fluorescence spectra were identified and intensities were measured at three pairs of excitation/emission wavelengths:  $\lambda_{ex}$ =280 nm and  $\lambda_{em}$ =625 nm,  $\lambda_{ex}$ =355 nm and  $\lambda_{em}$ =510 nm, and  $\lambda_{ex}$ =463 nm and  $\lambda_{em}$ =620 nm.



**Figure 10.** Agilent HPLC 1100. Image © Agilent technologies.

### *Calibration of pigment fluorescence using quinine sulphate*

To provide a quantitative measure of age-pigment in tissue, the fluorescence intensities that were measured from the extracted pigments were calibrated against the fluorescence of a standard solution range of quinine sulphate. Quinine in a dilute (0.05 M) sulphuric acid solution fluoresces strongly at an excitation wavelength ( $\lambda_{ex}$ ) of 350 nm and emission wavelength ( $\lambda_{em}$ ) of 450 nm. This characteristic allows very small quantities of quinine to be detected in the range that age-pigment was likely to be found (Se-Jong Ju, personal communication). The intensity of detected fluorescence is directly proportional to the quantity of

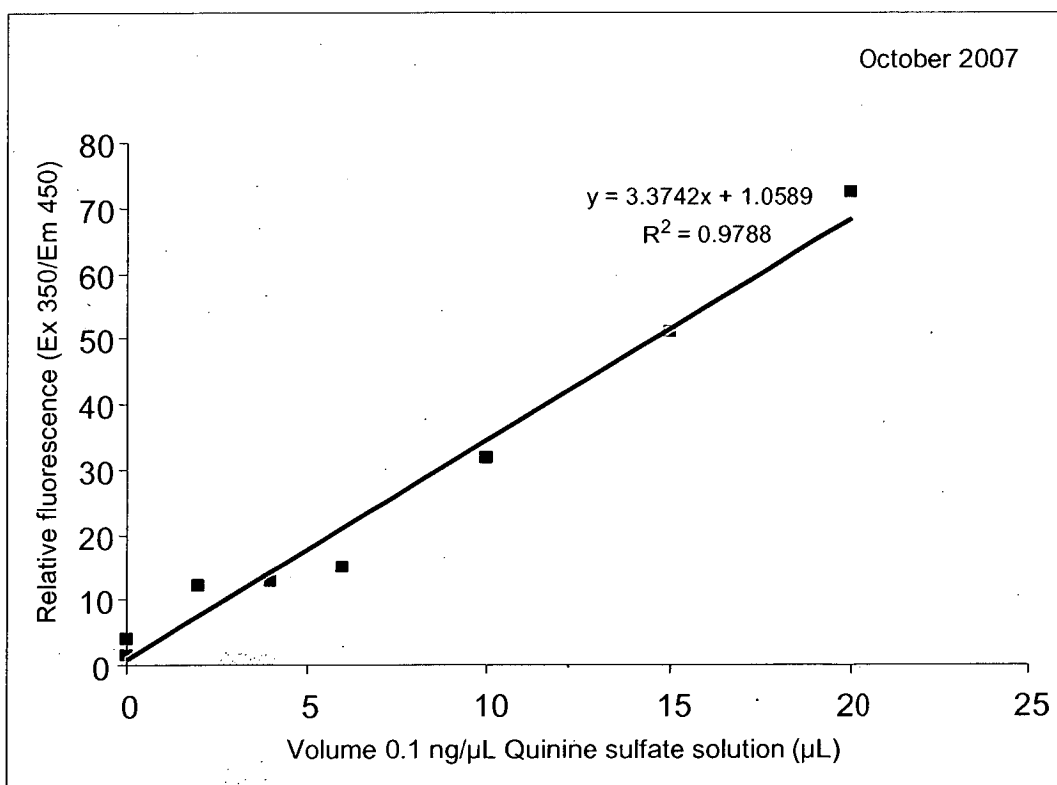
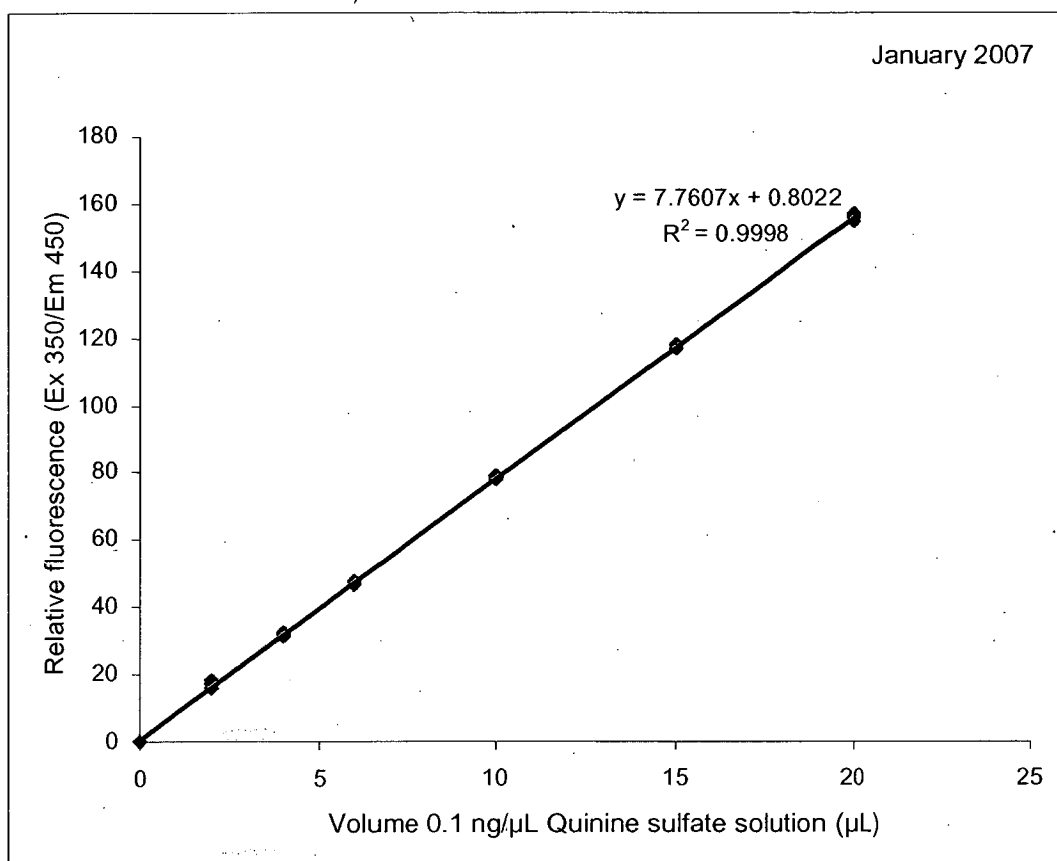
quinine in the sample; however, the measured intensities can vary significantly with small variations in method or conditions, for this reason, calibration curves were prepared from a range of known standard solutions.

Stock solution 1M sulphuric acid ( $\text{H}_2\text{SO}_4$ ) was prepared as follows: 56 ml of concentrated  $\text{H}_2\text{SO}_4$  was added to 500ml deionized water in a 1L beaker with stirring. 0.05M stock solution  $\text{H}_2\text{SO}_4$  was prepared by adding 100ml of the 1M stock solution to 500 ml of distilled water in a labelled, screw-capped reagent jug. The solution was mixed and diluted to 2.0L.

Preparation of the 1000 parts-per-million (ppm) (1000  $\mu\text{g/ml}$ ) quinine solution was as follows: 0.1207g quinine sulphate dihydrate was weighed and transferred quantitatively into a 100ml volumetric flask. A wash bottle containing distilled water was used to wash any remaining solid material from the weighing boat and the neck of the flask. A pipette was used to transfer 5.00ml of the stock 1M  $\text{H}_2\text{SO}_4$  into the flask. The quinine sulphate was dissolved in this sulphuric acid solution by swirling. Distilled water was then used to dilute to volume, and the contents of the flask mixed thoroughly. An intermediate 10 ppm quinine stock solution was prepared by transferring 5.0 ml of the 1000 ppm solution by pipette into a 500ml volumetric flask. 25.0ml of 1M  $\text{H}_2\text{SO}_4$  was added and the contents mixed and diluted to volume with distilled water. The stock solution was further diluted to give 0.1  $\mu\text{g/ml}$  (equivalent to 0.1  $\text{ng}/\mu\text{l}$ ) quinine sulphate solution. This was achieved by quantitatively transferring 1.00ml of the 10 ppm intermediate stock solution into a 100 ml volumetric flask and diluting to volume with 0.05M  $\text{H}_2\text{SO}_4$ . The stock solution 0.05M  $\text{H}_2\text{SO}_4$  was used as a blank.

Volumes of 2, 4, 6, 10, 15 and 20  $\mu\text{l}$  of quinine sulphate solution, equivalent to a range 0.2 to 2.0  $\text{ng}/\mu\text{l}$ , were injected by auto-sampler (Agilent 1100 series HPLC) with 0.05M  $\text{H}_2\text{SO}_4$  as the carrier solvent (0.8  $\text{ml min}^{-1}$ ). Fluorescence intensity was read at  $\lambda_{\text{ex}}=350 \text{ nm}$ ,  $\lambda_{\text{em}}=450 \text{ nm}$ . Calibration was undertaken on the day of measurement prior to measuring the fluorescence intensities of the extracted pigments, and then repeated at the end to make sure no changes, or 'drift', had occurred during the day.

A calibration curve was constructed by plotting the concentration of the quinine sulphate solution against signal strength (fluorescence intensity) (Figure 11). The regression of the relationship was used to determine the relative quantity of pigment in the krill eyestalk samples. The samples were analysed as two



**Figure 11.** Standard calibration curves of the fluorescence of quinine sulphate solution in 0.05M sulphuric acid. The calibrations were undertaken in January and October 2007, parallel to the analysis of the krill age-pigment samples.

batches at two different times, so the quinine calibration was run in parallel with each analysis in January and October 2007. Therefore two equations were developed from the individual regressions (Figure 11) for converting fluorescence to quantity of quinine, used as a proxy for quantity of fluorescent age-pigment.

### *Protein content*

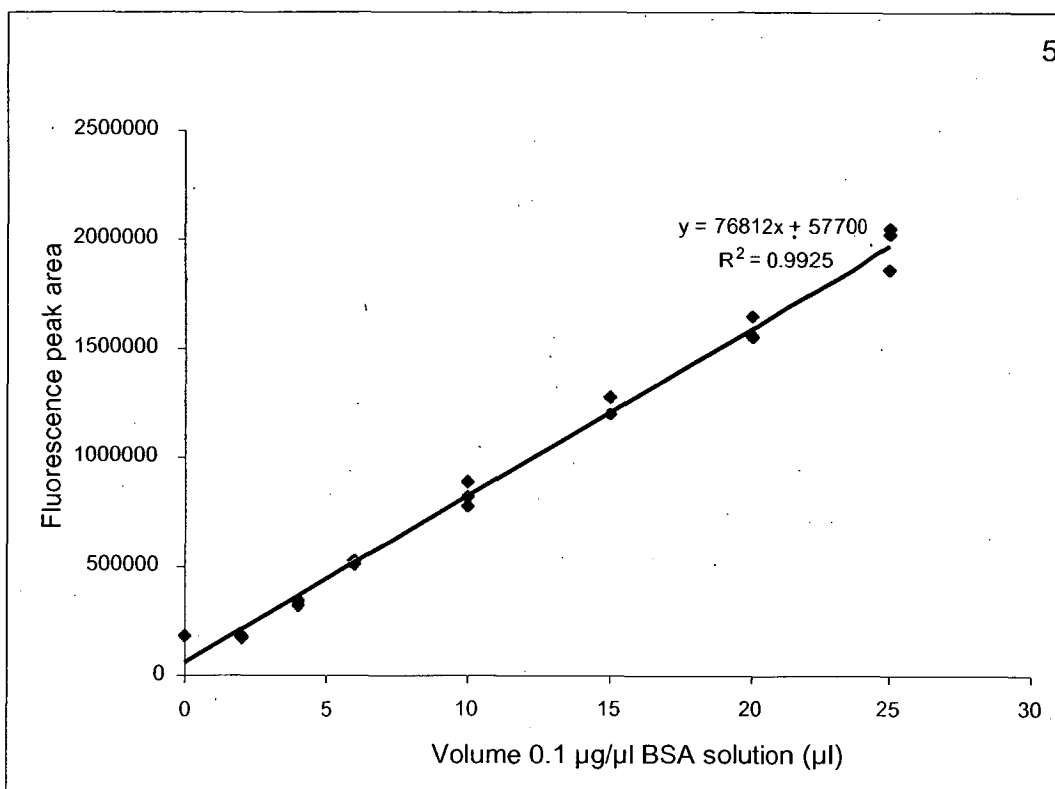
The second vial prepared during the pigment extraction (section 3.6 above) contained cellular debris, and was used to quantify protein content in the samples in order to standardise the amount of pigment measured. The total extract was dried under nitrogen (gas), and redissolved in 1 ml 0.16% v/w deoxycholic acid (DOC) solution (0.16g DOC dissolved in 100ml Milli-Q ultra-pure water) with sonication (30 W for 0.5 minute). The vials were then centrifuged at 800g for 10 minutes to pelletise the cellular debris, and 500 $\mu$ l of extract was then transferred to a new auto-sampler vial.

Volumes of 20 $\mu$ l from each protein extract were injected by auto-sampler (as above) with ultra-pure water as the carrier solvent (0.8 ml min<sup>-1</sup>) through the flow cell (volume 16  $\mu$ l). Fluorescence intensities were measured at  $\lambda_{ex}$ =280 nm,  $\lambda_{em}$ =345nm (gain/attn: 10/16) at a constant temperature of 20°C.

### *Protein calibration*

The fluorescence intensity of the extracted protein was calibrated using bovine serum albumin (BSA) as a standard. A stock solution of 0.1 $\mu$ g/ $\mu$ l BSA was prepared by dissolving 10.0mg BSA in 100ml 0.16% deoxycholic acid (DOC). Volumes of 2, 4, 6, 10, 15 and 20 $\mu$ l (equivalent to a range 0.2 to 2.0  $\mu$ g/ $\mu$ l BSA) were injected by auto-sampler (Agilent 1100 series HPLC) with Milli-Q ultra-pure water as the carrier solvent (0.8 ml min<sup>-1</sup>). Fluorescence intensity was read at  $\lambda_{ex}$ =280 nm,  $\lambda_{em}$ =345 nm by the fluorescence detector. As with the quinine calibration, the protein calibration was undertaken on the same days as sample analysis, which resulted in nine calibration curves.

Protein calibration curves were constructed from the concentration of the BSA solution against fluorescence intensity (Figure 12). Nine curves were



**Figure 12.** A representative plot (run 5) of a standard calibration curve of the fluorescence of bovine serum albumin (BSA) used for protein determination. Volume of solution injected directly translates to concentration of protein detected at fluorescence detector.

| Run | Regression equation  | R <sup>2</sup> |
|-----|----------------------|----------------|
| 1   | $y = 72220x + 30409$ | 0.9923         |
| 2   | $y = 69394x + 17519$ | 0.9957         |
| 3   | $y = 80929x + 46091$ | 0.9943         |
| 4   | $y = 79293x + 55543$ | 0.9901         |
| 5   | $y = 76812x + 57700$ | 0.9925         |
| 6   | $y = 74509x + 61022$ | 0.9924         |
| 7   | $y = 74090x + 52650$ | 0.993          |
| 8   | $y = 70256x + 87209$ | 0.9815         |
| 9   | $y = 70457x + 60905$ | 0.9907         |

**Table 3.** Regression equations derived from the bovine serum albumin (BSA) calibration, describing the relationship between fluorescence ( $y$ ) and quantity of protein ( $x$ ) in the sample.

prepared to accompany the sample protein analyses. The regression of the relationships was calculated (Table 3), and applied to the protein fluorescence

measured from the krill eyestalk tissue to determine the quantity of protein in the samples.

### *Standardisation*

The pigment fluorescence measured at each of the three sets of wavelengths was converted to quantify pigment amounts using the appropriate regression equation from the calibrations. Similarly, the quantity of protein in each sample was calculated from the protein fluorescence. The calculated quantity of pigment in the sample was standardized against the quantity of protein, to account for differences in body size and variability in tissue sampling.

## **3.8 Approach to statistical analyses**

The construction of the quinine and BSA calibration curves and regression analysis were undertaken in Microsoft Office Excel (Microsoft Corp. 1985-2001). For all data basic descriptive statistics (mean, standard deviation, and standard error) were calculated using Excel. Analyses and modeling of the multivariate data were carried out using the software package R and asreml (R Development Core Team, 2008).

Summary data, linear models with main effects, linear models with main effects and first order interactions, and Analysis of Variance (ANOVA) were calculated for each of the wavelength sets measured, hereafter denoted 'pigment 1', 'pigment 2' and 'pigment 3'. Tables of coefficients were prepared (see Chapters 5 and 6), along with graphs showing the nature of the interactions of the variables of temperature, diet, stress, sex and time (age) with the response variables pigment 1, pigment 2 and pigment 3, eye diameter, carapace length, wet weight eyes. The models were sequentially reduced based on significant effects.

By modeling the mean and variance in the real data for the known-age krill, a von Bertalanffy (VB) model was constructed and normally distributed populations for each age class of 1, 2, 3, and 4 yrs were simulated separately for each of two pigment classes, wet weight eyes and carapace length. Each of these predictor variables was grouped using 22 classes, and the frequency of animals in

each variable class by each age class was determined for each variable separately from the simulated data. These frequency data were then modeled using a form of ordinal regression called a continuation-ratio logistic regression, and from the fit of this model the proportion in each age class by variable was recovered (Candy, 1991, 2003). To determine how well each variable predicted age class, the percentage deviance explained by the continuation-ratio logistic regressions (Candy, 1991) was calculated where this statistic is the ordinal regression analogue of the familiar  $R^2$  statistic. Details of the analyses are provided within the results sections of Chapters 4, 5 and 6.





## Chapter 4: Diet-related mortality in captive krill

---

### 4.1 Abstract

The effect of diet and temperature on the mortality rate of a laboratory population of Antarctic krill, *Euphausia superba* Dana was investigated. Krill were kept in aquaria at the Australian Antarctic Division for a period of 280 days. The water temperature was maintained at either  $0 \pm 0.3^{\circ}\text{C}$  or  $3 \pm 0.4^{\circ}\text{C}$ , and the krill were given one of three diets: (a) food-limited, (b) continuously fed high levels of the phytoplankton *Phaeodactylum tricornutum* or (c) fed a mixed diet of *P. tricornutum* and a commercial preparation of the micro-algae *Schizochytrium* sp. (Algamac® 2000). The Antarctic Division's stock krill served as a control population and were kept in a 1000L tank in the dark at  $0 \pm 0.3^{\circ}\text{C}$  and fed high levels of the phytoplankton *P. tricornutum*.

Under control conditions the average mortality rate in krill was 1 in 1000 per day. Mortality rates increased significantly to 4.5 times, 3 times and twice as high as the control group when either food-limited, fed phytoplankton monoculture, or fed a mixed diet. Mortality was not significantly influenced by temperature or handling in the long-term (>90 days). Temperature did have an effect, however, on the absolute number of mortalities that occurred in the first 90 days of the experiment. In each diet group, more krill died at the warmer temperature of  $3^{\circ}\text{C}$  than at  $0^{\circ}\text{C}$ . As the effects of diet (lack of nutrition) were more important in determining mortality after this time, the temperatures effects appeared to diminish with time. The well fed krill on either diet exhibited excellent survivorship for the first 90 days, especially at the colder temperature, however after this time the krill on the mixed diet were better able to resist disease and handling stress than the krill that were fed phytoplankton alone. The long-term survivorship of krill was significantly improved by giving krill a mixed diet.

## 4.2 Introduction

The krill *Euphausia superba* is an integral component of the Antarctic ecosystem. There is ongoing scientific interest in this keystone species, which is also the target of a fishery (El-Sayed, 1994; Nicol, 2000). Krill are difficult to study in the wild due to the remoteness of the Antarctic region, the severity of environmental conditions and the disturbance that ships have on natural conditions (Brierley *et al.*, 2002). In good conditions, some *in situ* observations are possible by SCUBA divers (Hofmann *et al.*, 2002) or underwater vehicles with video imaging or echosounder equipment (Brierley *et al.*, 2002), but it is impossible to observe krill in the ocean for extended periods of time. Fundamental questions about the biology of krill can therefore be investigated by research undertaken on krill kept alive in captivity. For this reason most research on living Antarctic krill is conducted in the laboratory (Nicol, 2000), and live krill have been maintained at the Australian Antarctic Division since 1981 (King *et al.*, 2003) for this research.

The collection of live krill from the wild for experimental studies is costly and logistically complex. It is therefore especially desirable that krill which are collected for research purposes are kept alive for as long as possible. Krill can survive for several years when maintained in groups (Rob King, personal communication), and as long as 6-7 years as intensively-cared for individuals (Ikeda *et al.*, 1985; Ikeda & Thomas, 1987). It is unknown, however, whether captive krill have a life-span similar to wild krill.

Optimal conditions are difficult to reproduce in the laboratory, and captive krill do not grow as large as wild krill. For example, captive krill generally do not grow much beyond a mean length of 33mm (McGaffin *et al.*, 2002). Conversely, krill that are large when they are collected can shrink in captivity to a similar mean length of 29mm (Sun *et al.*, 1995), 33mm, (Sun, 1997) or 35mm (McGaffin *et al.*, 2002), depending on conditions. In comparison, wild adult krill reach modal length classes around 30-40mm, 46-48mm, and 52-54mm (Reid *et al.*, 2004).

The Commission for the Conservation of Antarctic Marine Living Resources (CCAMLR), which manages the krill fishery, has standard protocols for the treatment of length data on Antarctic krill, and classifies wild krill that are smaller than 35mm long as juvenile (Miller & Trivelpiece, 2007). Clearly, this is

problematic if it is known from laboratory-kept krill that larger, older and mature krill can fall into this 'juvenile' size-class following a period of time kept in apparently sub-optimal conditions. It is therefore crucial that captive krill are given conditions in which they can grow comparably with their wild counter-parts, and experience a similar life-span, in experiments that aim to provide generalisations to field conditions.

The aim of this study was to investigate the role of diet and temperature in mortality of captive krill, with the supplementary goals of keeping krill alive for as long as possible and for stimulating growth comparable to natural rates (see Siegel, 1987; Poleck & Denys, 1982). These studies were initiated to provide an experimental underpinning for the age determination research in the following chapters.

#### 4.3 Materials and methods

Live Antarctic krill *Euphausia superba* were collected from the ice edge at 65° 8.2'S, 109° 42.15'E at night on the 26<sup>th</sup> March 2001. Sampling was conducted from the RSV *Aurora Australis*. Krill were caught using a Rectangular Mid-water Trawl (RMT 8) net towed for 10 minutes at a depth of 0-30 metres. Live krill were transferred from the net to refrigerated flow-through tanks. The krill were maintained onboard ship for 10 days during the return to Australia, as described previously (King *et al.*, 2003), at which time they were counted and transferred to a 1000 litre aquarium at the Australian Antarctic Division headquarters in Tasmania, Australia. The krill were kept in the aquarium in the dark at approximately 0°C and fed *ad libitum* a culture of *Phaeodactylum tricornutum*, to allow the stress-induced mortality rate to subside. The aquarium was checked daily and all mortalities were removed and counted.

After two months a sub-sample of 600 krill was randomly selected from the tank and divided evenly between twelve 20 L containers to give 50 krill per container at a stocking density of one krill per 330 ml. Six of the containers were placed in a refrigerated laboratory at  $3 \pm 0.4$  °C – referred to as the 'warm' treatment, and the other six containers were placed in a laboratory kept at  $0 \pm 0.3$  °C – referred to as the 'cold' treatment. The water temperature in each container was

measured digitally and recorded daily. The containers were checked daily for exuviae and mortalities. Mortalities were recorded and dead krill were removed, placed within a labelled cryotube and frozen in liquid nitrogen for later measurement. Approximately half of the water in each container was replaced daily with fresh, chilled seawater, to improve the oxygen saturation of the water and remove nitrogenous waste and excess food. The stocking density was maintained throughout the course of the experiments, so that if two krill died and were subsequently removed from a container, the water volume was reduced by 2 x 330 ml.

Immediately following the water change, the krill were fed according to one of three diets; at each temperature the krill in two containers were food-limited, two were fed high levels of phytoplankton, and two were fed a mixed diet of phytoplankton and a commercial preparation of re-hydrated spray-dried algal cells (Algamac-2000®) purchased from Aqua-fauna Bio-marine. The phytoplankton used was a laboratory culture of the diatom *Phaeodactylum tricornutum*, which was cultured as described by King *et al.* (2003). The krill that received high levels of phytoplankton were given a concentration of approximately  $6.08 \times 10^4$  cells ml<sup>-1</sup> daily. The krill that were fed the commercial preparation of algal cells received 5.13 mg l<sup>-1</sup> dry weight of *Schizochytrium sp.* daily, which provided a saturating quantity of protein, as recommended by the manufacturers for the growth of cultured shrimp. The *Schizochytrium sp.* preparation was reconstituted in chilled seawater. An electric mixer was used for the first three months of the experiment, however this method did not break the powder into very small or even-sized particles, and over time the algal cells accumulated on the krill's setae. The preparation was altered to agitating the Algamac-2000® in seawater in a closed container by hand for approximately 1 minute, as this was found to provide a more consistent particle size solution. The amount offered to the krill was also reduced by more than half to 2.0 mg l<sup>-1</sup> as the initial concentration was found to be far more than the krill could eat, and fouled the containers quickly. This result was supported by concurrent experimental feeding work that showed a saturating diet for krill is about 2mg l<sup>-1</sup> protein day<sup>-1</sup> (Alonzo *et al.*, 2005).

The krill on the food limited diet were fed a small amount of phytoplankton once a week. The quantity of phytoplankton was arbitrarily decided to be  $1.52 \times 10^4$

04 cells per ml, once per week, which was 25% of the concentration of phytoplankton that the well-fed krill received on a daily basis, or less than 4% of the total phytoplankton concentration made available to the well-fed krill. Additionally, the sea water, which was filtered to 0.45  $\mu$ l, may have contained some natural biota on which the krill may have fed.

The krill in half of the experiments, one container for each of the temperature x food treatments, were briefly removed from their aquaria every 30 days and photographed alive using a Sony video camera attached to a compound microscope. Length measurements were then taken at a later time using digital image analysis (Sigma-ScanPro software by Jandel Corp., 1993-1995). Carapace length was measured as the length from the tip of the rostrum to the mid-dorsal posterior edge of the carapace (Standard 4, Kirkwood, 1982), and eye diameter measured (data not shown). In order to minimise handling time gender was not observed for the live krill. To control for handling effects on long-term growth and longevity, the krill in the remaining experiments were not handled, and were not measured until they died and were subsequently removed from the aquaria.

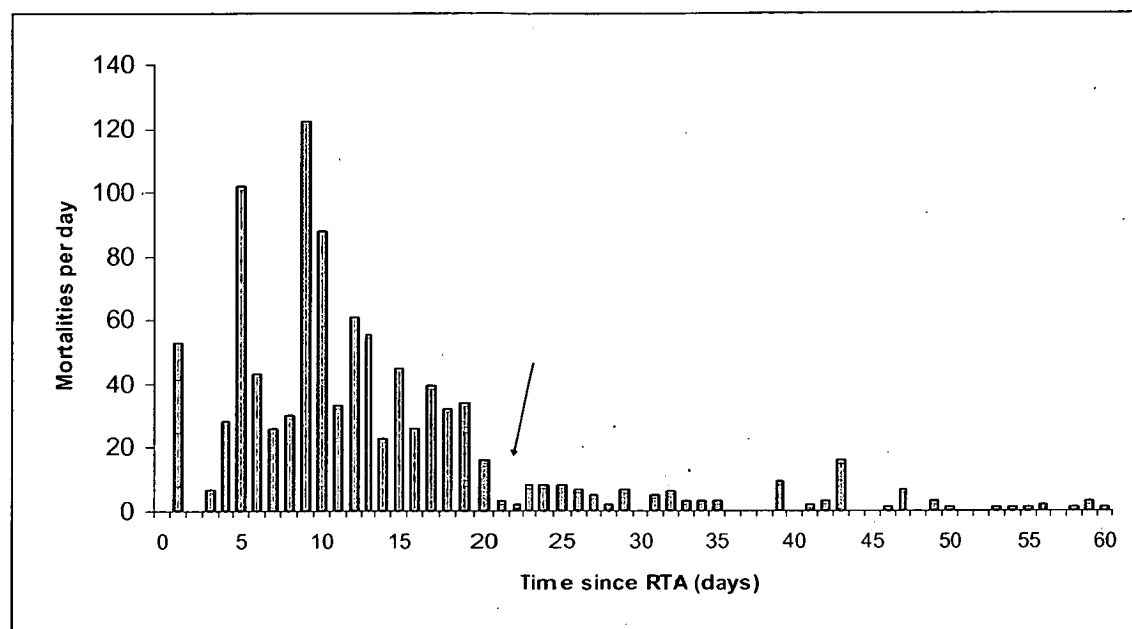
## 4.4 Results

### *Population size and initial mortality rate*

The initial population size of captive live krill was estimated to be ~3000 on the day of return to Australia (RTA). The mortality rate for the captive krill in the 60 days following RTA peaked at 120 deaths (4% of the population) on day 9 (Figure 13). It took 20 days for the mortality rate to drop to a low and stable level of  $3 \pm 0.6$  deaths per day. This is equivalent to a mortality rate of  $0.10 \pm 0.02\%$  (SE) in a population of 3000 per day (as indicated by the arrow on Figure 13). The initial high mortality rate was attributed to damage done to the thoracic appendages in the net at the time of capture, probably due to the weight of the catch, which prevented the krill from successfully moulting and ultimately resulted in premature death.

Survivorship in the aquarium improved once all captive krill had been through one moult cycle. This occurred after 30 days (10 days on ship plus 20 days in aquarium); the maximum intermoult period from Ikeda *et al.*, 1985 is 29.8 days.

The krill were transferred into the feeding experiments after the mortality rate had been stable for a week.

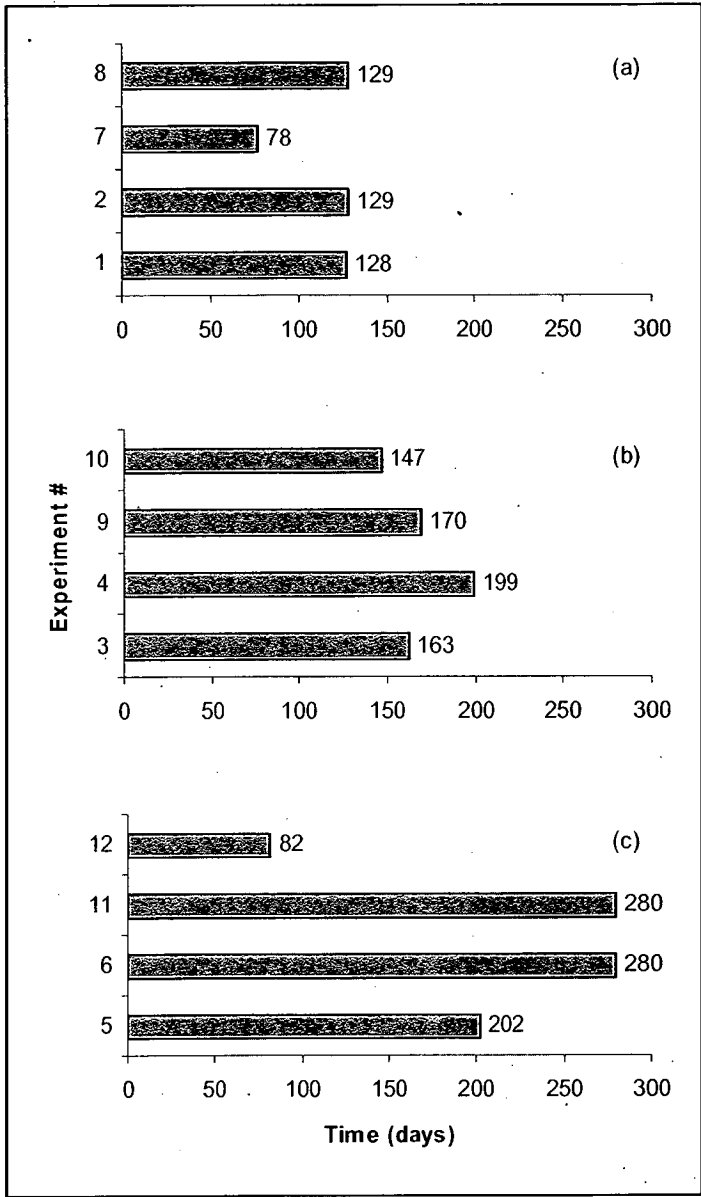


**Figure 13.** *Euphausia superba*. Mortality rate in a population of 3000 live krill after return to Australia (RTA). Arrow indicates point at which it was calculated that all krill would have moulted once following capture and mortality rate became stable.

#### *Time taken to reach 50% mortality*

The population 'half life' was calculated as the number of days taken to reach 50% mortality in the original population of krill in each experiment (Figure 14). There was a highly significant difference between the mortality rate of krill that were food limited, fed a phytoplankton monoculture and fed a mixed diet (ANOVA:  $F_{2,8} = 17.685$ ,  $p = 0.001$ ). The food-limited krill (experiments 1, 2, 7 & 8) (Figure 14a) reached 50% mortality in 78 – 129 days (mean  $\pm$  SE =  $116 \pm 13$ ,  $n = 4$  populations). The krill fed a phytoplankton monoculture (experiments 3, 4, 9 & 10) (Figure 14b) had better survivorship, reaching 50% mortality in 147-199 days ( $170 \pm 11$  d,  $n = 4$  populations). The krill fed a mixed diet of phytoplankton and Algamac<sup>®</sup> 2000 (experiments 5, 6 & 11) (Figure 14c) had the best survivorship, reaching 50% mortality in 202-280 days ( $254 \pm 26$  d,  $n = 3$  populations). There were high numbers of deaths in experiment 12 (Figure 14c) on days 73 (15 krill) and 82 (12 krill). The mortalities on these two days accounted for over 50% of the

original population of krill in that experiment. It is unlikely that these mass mortalities were related to the experimental conditions being tested, and therefore this experiment was subsequently excluded from analysis.



**Figure 14.** *Euphausia superba*. Number of days to reach 50% mortality in the original population of krill in each experiment, when (a) starved, (b) fed a live phytoplankton monoculture, and (c) fed a mixed diet of live phytoplankton and a commercial preparation of spray dried algal cells (Algamac® 2000).

The time taken to reach 50% mortality in each of the three diet categories was significantly different (ANOVA:  $F_{2,8} = 17.685$ ,  $p = 0.001$ , Table 4). The data are shown converted to mortality rate (as a percentage of the original population size in each group, per day) so that they could be compared with the mortality rate



in the control population from which they were sourced. The difference in mortality rate between each diet group and the control population was highly significant (ANOVA:  $F_{3,47} = 14.846$ ,  $p < 0.001$ ). Starved krill had a mortality rate 4.5 times that of the source population, whereas krill fed phytoplankton had a mortality rate three times that of the source population, and krill fed a mixed diet had twice the mortality rate of the source population. Experiments in each of the diet groups were conducted at temperatures of  $0 \pm 0.3^{\circ}\text{C}$  or  $3 \pm 0.4^{\circ}\text{C}$ , but as temperature was found to have no statistically discernable effect on the amount time taken to reach 50% mortality, the data were pooled for the above analysis.

| Diet          | Mean # days to reach 50% mortality<br>$\pm$ standard error | Mortality rate (% population/day)<br>$\pm$ standard error | n         |
|---------------|------------------------------------------------------------|-----------------------------------------------------------|-----------|
| Food limited  | $116 \pm 12.7$                                             | $0.45 \pm 0.06$                                           | 4 (expts) |
| Phytoplankton | $170 \pm 10.9$                                             | $0.30 \pm 0.02$                                           | 4 (expts) |
| Mixed diet    | $254 \pm 26.0$                                             | $0.20 \pm 0.02$                                           | 3 (expts) |
| Control popn  |                                                            | $0.10 \pm 0.02^*$                                         | 40 (days) |

**Table 4.** *Euphausia superba*. Average number of days in which 50% mortality occurred in three populations of Antarctic krill that were fed different diets. Data are also shown converted to mortality rate (as a percentage of the original population size in each group, per day) for comparison with the control population. \*Calculated from a mean mortality rate of  $3 \pm 0.6$  deaths per day in an estimated population of 3000, in days 21-60 after RTA (see Figure 13).

### *Temperature effects*

In the first 90 days temperature was found to have a significant effect on mortality (Table 5). Significantly more krill died in the experiments conducted at  $3^{\circ}\text{C}$  than those that were kept at  $0^{\circ}\text{C}$ , in the first 90 days (two factor ANOVA:  $F_{2,1} = 22.231$ ,  $p < 0.05$ ).

### *Handling effects*

The krill in odd-numbered experiments were handled every 30 days while measurements were taken. Handling the krill did not have a measurable effect on

any of the population mortality rates (Fig. 14). For the above analyses, therefore, the 'handled/not handled' data was either pooled or used as replicate experiments.

| Diet          | # Mortalities |          |
|---------------|---------------|----------|
|               | T = 0°C       | T = 3°C  |
| Low food      | 42 (100)      | 47 (100) |
| Phytoplankton | 2 (100)       | 6 (100)  |
| Mixed         | 10 (100)      | 18 (100) |
| Total         | 54 (300)      | 71 (300) |

**Table 5.** *Euphausia superba*. Number of mortalities in a 90 day period in 6 populations of 100 krill. Krill were kept at 0°C and 3°C, and fed one of 3 diets: (a) low food, (b) a phytoplankton monoculture, or (c) a mixed diet of phytoplankton and Algalmac® 2000.

## 4.5 Discussion

Mortality rates for the krill in the diet/temperature experiments were calculated from the time taken for half of each population to expire – the 'population half-life'. This parameter was chosen to demonstrate mortality at a population level, and to even out the irregular mortality of individual krill during the course of the experiments. This is particularly important for observing the effect of experimental conditions on a population, and to remove the effects of moult-synchrony on mortality. Mortality seemed to be partly coupled to moulting: incompletely shed exuviae were observed on a number of the dead krill, and failure to moult properly appears to be associated with the cause of death. As moulting can be synchronized within a krill population (Buchholz, 1991) this effect has the potential to create mass mortality events.

Krill that are food limited in the laboratory are capable of surviving for a relatively long time. Figure 2 shows that half of a captive population can be expected to be alive after up to 129 days on a food-limited diet. This is consistent with the results of a previous study which found that individual krill could live for as long as 211 days when starved (Ikeda & Dixon, 1982). The mortality rate of food-limited krill in the present study is  $0.45 \pm 0.06$  % krill per day, (9 mortalities per day in 2000 krill). The rate recorded is much higher than the mortality rate of  $0.10 \pm 0.02\%$  (two krill in 2000) of the control population being held in standard conditions at the Australian Antarctic Division. Although krill are capable of

surviving periods of food limitation, this ability has probably developed as an adaptation to seasonally limited food availability, however, prolonged lack of food will eventually result in death.

It must be assumed that in these experiments the krill may also have had access to some food in the sea-water used in the experiments. The sea water was pre-filtered to 0.45  $\mu\text{m}$  at the time of collection, but due to pore-size variability this did not completely exclude all larger particles (Rob King, Australian Antarctic Division, personal communication 2008). Krill will consume a wide range of food types and particle sizes (Nishino & Kawamura, 1994), and it is possible that the filtered sea-water contained some pico-plankton that the krill could ingest (Klaus Meiners, Antarctic Climate and Ecosystems Cooperative Research Centre, personal communication 2008). It has been reported that Antarctic krill prefer food particles in the size range of 20-40  $\mu\text{m}$  (Opalinski *et al.*, 1997). If present in the sea water collected for their maintenance, food particles of this size would not have been removed by the filter.

Cannibalism was observed under food-limited conditions and provided another source of food to individual krill. Partially eaten krill were removed whenever they were found, however it is difficult to calculate how much nutrition these may have contributed to the diet. Cannibalism has been observed in other laboratory studies (McWhinnie & Marciniak, 1964) and has been suggested as an overwintering strategy in krill but the degree to which it is widely practiced in the wild is unknown (Nishino and Kawamura, 1994).

Very few of the krill that were fed high levels of the phytoplankton *Phaeodactylum tricornutum* died in the first 90 days (Table 2). Only two mortalities occurred in the krill populations that had been kept at 0°C and fed high levels of phytoplankton (n=100 krill). In the short-term (<90 days) this appears to be the most optimal of the conditions tested for maintaining krill in the laboratory. This result, however, did not translate through to the 'half-life' of the population. Figure 2 shows that krill fed a mixed diet had a significantly better survival rate in the long term than the food-limited krill or those fed a phytoplankton monoculture. It is probable that this is due to nutrition; the mixed diet provided a more diverse range of nutrients in the diet of the krill. The commercialised heterotrophically grown spray-dried marine algae, *Schizochytrium sp.* (Algamac-2000, Aquafauna Bio-Marine, Los Angeles, CA.) was chosen as a food supplement because it has

shown 'good acceptability and performance' when used as an algal replacement for feeding penaeid shrimp (Rosenberry 1996). *Schizochytrium sp.* has nutritional qualities which make it suitable as a diet supplement in aquaculture, particularly to provide the fatty acid docosahexaenoic acid (DHA). This is an essential fatty acid, which cannot be manufactured in the body and must be obtained through diet. In crustaceans this omega-3 fatty acid is required in the diet for proper growth and moulting (for example Lim *et al.*, 1997; Sheen & Wu, 2002). The live alga *Phaeodactylum* contains eicosapentaenoic acid (EPA) but low or negligible amounts of DHA (Molina Grimal *et al.*, 1996).

The krill fed a mixed diet suffered much higher initial mortality than the krill fed phytoplankton alone. Microscopic examination of the krill that had died which had been fed a mixed diet, revealed that they had clogged setae and gills from the large amount of *Schizochytrium* being provided to them, and the food particle size was possibly too high. Suspended particulate matter at high levels can cause clogging and may be fatal for filter feeders, (Pakhomov *et al.*, 2003). High densities of algal colonies can also cause clogging of the feeding-basket (Pieters, 1980; Haberman *et al.*, 2003). For this reason the preparation and amount of Algamac was altered as outlined in the methods section at this time, and this reduced long-term mortality.

There was higher mortality at 3°C than at 0°C for all diet groups in the first 90 days. In laboratory experiments, water temperature significantly effects metabolism, growth rate and intermoult period (IMP) (e.g. Poleck & Denys, 1982; Nicol & Stolp, 1991; Buchholz, 1991; Iguchi & Ikeda, 2005; Tarling *et al.*, 2006). Temperature effects on mortality, however, are not well documented. Krill naturally inhabit a range of temperatures from -1.8 to 5°C (Quetin *et al.*, 1994). The observed higher mortality rate in these experiments at 3°C, which falls within this range, was unexpected. One study reported krill placed in 4°C "became quiescent, lost their glassy transparency and died within 24 hrs" (McWhinnie & Marciniak, 1964). The authors, however, discuss high mortality in all of their experiments for *E. superba*, which they attribute to the species' lack of tolerance to temperature changes, and the difficulty of maintaining this species in a laboratory (McWhinnie & Marciniak, 1964). In contrast, Hirche, (1984) found no mortality related to higher temperature acclimation in a 30 hour experiment. In the present

study the increased mortality observed at 3°C compared with 0°C was therefore probably a result of increased rate processes, combined with less optimal conditions for the krill.

Diet was clearly the most important factor governing krill mortality rates in this study. The time taken to reach 50% mortality was significantly affected by the diet of the krill in the three groups. Two earlier studies (Ikeda & Dixon, 1982; Shin & Nicol, 2002) showed that krill could survive successfully for 195 days (Shin & Nicol, 2002) or 211 (Ikeda & Dixon, 1982) without food. Unlike this study, Ikeda & Dixon (1982) and Shin & Nicol (2002) were working with small groups of krill that were maintained individually, and which were replaced in the experiment as mortalities occurred. Results found for small groups of individually kept krill do not necessarily reflect what will happen in larger groups of aquarium-maintained krill, which was the approach used in this study. The perceived stress of being handled for measurements didn't cause any significant additional mortality in those populations.

Estimates of natural mortality rates have been obtained from various methods, and range widely from  $M = 0.38 - 5.5$  for post larval krill (Pakhomov, 1995b; Siegel & Nicol, 2000). Based on realistic longevity estimates of 6-7 years, however, mortality estimates fall in the range  $M = 0.66 - 1.35$ , which corresponds to a survival rate of less than 51% from one year to the next for adult krill (Knox, 1993). Mortality rates are thought to vary inter-annually and geographically as well as between age classes and as a result of predator selection (Siegel & Nicol, 2000). Mortality of wild krill is much higher than for the captive krill in the present study which is not surprising because under experimental conditions, krill were isolated from predation, parasites and disease which are likely to be the major sources of mortality in wild krill. Mortality is inversely related to longevity and directly related to growth rate (Hoenig, 1983). It might be assumed, therefore, that the krill in this study might have lower growth rates and increased longevity compared with wild counterparts. Further studies on the mortality rate of krill under more natural food conditions might enable the calculation of an intrinsic mortality rate which could be subtracted from field estimates of mortality to provide an estimation of extrinsically imposed mortality.

Krill inhabit an environment that is physically heterogeneous over large temporal and geographical scales. Food availability, daylight cycles and

temperature are strongly seasonal. In the natural environment krill may encounter water temperatures as much as six degrees in difference and very small temperature differences may exert a very strong influence on the physiology of krill. Modeling of krill populations at South Georgia and the Antarctic Peninsula suggest that local environmental conditions strongly influence the growth of krill (Reid *et al.*, 2004).

It is becoming increasingly important to maintain study organisms within artificial environments. This is especially true for animals such as Antarctic krill that are difficult to obtain or study in the wild. Recent advances in husbandry partly stem from recognition that krill have more complex requirements than once thought. Krill require a variety of food, light:dark cycles and possibly temperature cycles, to mimic natural conditions (Teschke *et al.*, 2007; Teschke *et al.*, 2008). They have complex nutritional requirements, and probably have access to a wide range of food types in the wild (Atkinson & Snyder, 1997). Krill diet changes seasonally, and is diverse and adaptable (Passmore *et al.*, 2006; Martin *et al.*, 2006). Krill can consume a variety of food items ranging from phytoplankton and sea ice algae to copepods, other krill, faecal pellets, eggs, marine snow and bacterial aggregations (Perissinotto *et al.*, 2000). It is hardly surprising then that a laboratory diet of a single phytoplankton species does not satisfy the nutritional requirements of krill, despite evidence that they have a physiological adaptation to periods of starvation. Their long-term survivorship in captivity may not be just a question of how much food the krill are offered, but the quality of the food, which becomes of importance for ageing research.

## **4.6 Conclusions**

This study was initiated through a need to keep krill alive for extended periods for ageing research, and to have them grow at a rate comparable to natural rates. The findings of this study show that the longevity of krill can be improved by introducing nutritional variety to their diet. Temperature and handling had a lesser effect on the survival of krill than did food quantity and quality.



## Chapter 5: Quantification of extractable pigments in Antarctic krill of known age

---

### 5.1 Abstract

Antarctic krill, *Euphausia superba*, were hatched from eggs in the laboratory and maintained for 4 years. This population was sampled periodically to provide known-age krill for age-pigment analysis. Extractable pigments from the eyestalk ganglia and eyes of krill were quantified using fluorescence intensity, and standardised against a measure of protein in each sample. Three peak fluorescence intensities were measured at wavelengths of excitation 280nm, emission 625nm (pigment 1); excitation 355nm, emission 510nm (pigment 2); and excitation 463nm, emission 620 nm (pigment 3), and standardised against the quantity of protein in each sample. A standard quinine sulphate calibration was used to determine quantity of pigment from fluorescence intensity. There was a positive correlation between the quantity of pigments 1 and 3 and the chronological age of Antarctic krill over four years post-hatching. The accumulation rate of these pigments was dependent on sex. A model was developed to use pigment 3 to predict age, which was tested against the real data. Quantity of extracted pigment predicted age better than krill body length or eye size, both of which have previously been used as proxies for age. For mature adult krill, this method using pigments can discriminate between krill of a similar size aged either 12 or 36 months old. The increase in quantity of pigments appears to slow with increasing age, and for krill the increase in pigments from 36 months to 48 months was small. The large amount of variation in pigments between animals of a similar age is attributed to natural variance based on biological factors that affect metabolism, and variation in experimental conditions. The use of extracted age-pigments provides an improved tool for age estimation in Antarctic krill, particularly if used in conjunction with other demographic information.



## 5.2 Introduction

In crustacean fisheries biology there are many circumstances where a technique for age estimation would be valuable (Smith & Addison, 2003). Unlike fish and molluscs, which have permanent hard parts like vertebrae, otoliths, statoliths, fins, shells, and scales that accumulate age rings (Weisberg, 2003), crustaceans such as Antarctic krill moult periodically (e.g. Buchholz, 1991) and hence shed any external physical evidence of ageing. In order to establish population age structure, accurate age determination is essential, particularly for the sustainable management of exploited species such as krill. The apparent lack of the physical evidence of ageing in Antarctic krill presents specific difficulties. Further to this, the important position this species holds in the wider marine ecosystem means that the accurate understanding of *Euphasia superba* demography is critical. Currently, age estimations are combined with information about growth and mortality and this is used as a tool for sustainable resource management (Nicol, 1990).

Using fluorescent pigments to determine the age of krill has been suggested as a solution to the issue of age determination (Ettershank, 1984). Age-pigments are the autofluorescent end-products of metabolic biochemical reactions between oxidised lipids and proteins (Yin 1996). Over time, these pigments accumulate in post-mitotic tissue, such as neurons. The hypothesis was put forth that by measuring pigment accumulation a correlation can be made with biological, and perhaps chronological, age (Brunk & Terman, 2002).

An age estimation method based on measuring age-pigments in histological thin sections has been used for some larger crustaceans such as lobsters (e.g. Sheehy *et al.*, 1998), but is difficult to apply to small crustaceans like krill (Bluhm *et al.*, 2001). This method is labour-intensive, and it is difficult for large sample sizes. Using small pieces of tissue presents difficulties in achieving consistent orientation of samples in the paraffin (personal observation), something that is vital for quantification. An alternative method based on extracting pigments has been developed, and has been used with some success for the blue crab *Callinectes sapidus* (Ju *et al.*, 1999, 2001, 2003). Pigment extraction allows for larger sample sizes to be studied, and also provides a means of examining small crustaceans,

such as krill, that have thus far not been amenable to the histological technique (Sheehy, 1990; Bluhm *et al.*, 2001).

Accurate age estimation from pigments requires knowledge of accumulation rates in known-age animals. In the present study, pigment extracted from a population of reared Antarctic krill, *Euphausia superba*, were used to produce a calibration curve of age-pigment with chronological age. Secondly, a data simulation model based on observed pigment values was created to test how well extracted pigments predict age in krill. Thirdly, pigment-based age prediction was compared with various morphological features, such as length and eye size, that are more traditionally used to predict age in krill.

### 5.3 Materials and methods

A population of known-age Antarctic krill was raised from eggs spawned in captivity in 2000 and 2001 at the Port of Nagoya Public Aquarium, Japan. They were maintained over a period of four years at 0°C, and sampled at time intervals of 30 days, 1 year, 3 years and 4 years post-hatching. The larvae from Calyptopis to Furcilia III-IV maturity stage were kept in 25 litres seawater in 30L rectangular styrene tanks. From Furcilia IV-V stage to adult maturity the krill were maintained in 200L round polycarbonate tanks. The larval krill were stocked at a density of either 40 individuals per litre (population hatched in 2000) or 20 individuals per litre (population hatched in 2001). Juvenile stage krill were stocked at a density of 1 individual per litre. The krill were fed 10,000 cells/ml *Phaeodactylum tricornutum* (final concentration) twice a day, plus a ratio of one brine shrimp *Artemia salina* per larvae. The adult krill were fed and maintained as described by Hirano *et al.*, (2003); Hirano & Matsuda, (2003). The water in the aquaria was filtered at a rate of total volume 5 times per day. 25 x Calyptopis-stage (1 month-old) krill, 25 x 1 year-old krill, 12 x 3 year-old krill, and 12 x 4 year-old krill were sacrificed by placing them within a cryotube which was then frozen in liquid nitrogen. The samples were stored at -86°C for later analysis.

The krill were defrosted by transferring the cryotubes to -20°C for approximately 20 minutes, and then into an ice/water slurry for several minutes. Each krill was then removed from the cryotube by flushing with chilled sea water

into a glass Petri dish. Carapace length, eye diameter and sex of the krill were recorded as described in Chapter 3. The eyestalks were excised from the defrosted krill, so that the eyestalks remained attached to each other at the base. The extraction method used was modified from Ju *et al.*, (1999). The eyestalks, including eyeballs, were momentarily blotted and weighed in a tared 2 ml amber glass HPLC vial. 1 ml of mixed solvent DCM-methanol (2:1 v/v) was added to the vials, which were then capped. The vials were sonicated (40W) for 1 minute to aid extraction of soluble pigments, and then centrifuged at 800g for 10 minutes at 4°C to remove cellular debris. Half of the extract (500 µl) was quantitatively transferred to a glass vial insert in an amber glass vial, and the pair of vials dried under liquid nitrogen. Dried samples were redissolved in 250 µl of methanol and stored at -86°C until all samples were extracted in preparation for the fluorescence assay.

Volumes of 20µl from each pigment extract were injected by auto-sampler (Agilent 1100 series HPLC) with methanol as the carrier solvent (0.8 ml min<sup>-1</sup>) through the flow cell (volume 16 µl). The major fluorescence spectra were identified and intensities were measured at three pairs of excitation/emission wavelengths:  $\lambda_{ex}=280$  nm and  $\lambda_{em}=625$  nm,  $\lambda_{ex}=355$  nm and  $\lambda_{em}=510$  nm, and  $\lambda_{ex}=463$  nm and  $\lambda_{em}=620$  nm, hereafter denoted pigment 1, pigment 2 and pigment 3, respectively. To provide a quantitative measure of age-pigment in tissue, the fluorescence intensities that were measured from the extracted pigments were calibrated against the fluorescence of a standard solution range of quinine sulphate (see Chapter 3).

The second vial prepared during the pigment extraction (see above) contained cellular debris, and was used to quantify protein content in the samples in order to standardise the amount of pigment measured. The total extract was dried under nitrogen (gas), and redissolved in 1 ml 0.16% v/w deoxycholic acid (DOC) solution (0.16g DOC dissolved in 100ml Milli-Q ultra-pure water) with sonication (30 W for 30 seconds). The vials were then centrifuged at 800g for 10 minutes to pelletise the cellular debris, and 500µl of extract was then transferred to a new auto-sampler vial. Volumes of 20µl from each protein extract were injected by auto-sampler (as above) with ultra-pure water as the carrier solvent (0.8 ml min<sup>-1</sup>) through the flow cell (volume 16 µl). Fluorescence intensities were measured at  $\lambda_{ex}=280$  nm,  $\lambda_{em}=345$ nm (gain/attn: 10/16) at a constant temperature

of 20°C. The fluorescence intensity of the extracted protein was calibrated using bovine serum albumin (BSA) as a standard (see Chapter 3). The calculated quantity of pigment in each sample was standardized against the quantity of protein, to account for differences in body size and variability in tissue sampling.

The carapace length, wet weight eyes and pigments 1 and 3 were log transformed and plotted against age data to construct growth curves using the von Bertalanffy (VB) equation:

$$L = L_{\infty} \{1 - \exp[-\kappa (A - t_0)]\}$$

where  $L$  is carapace length or wet weight eyes or pigment values,  $A$  is age,  $L_{\infty}$  is the upper asymptote,  $\kappa$  is the rate parameter and  $t_0$  is the location parameter (Appendix 1).

Further, by modeling the mean and variance in the real data for the known-age krill, a von Bertalanffy (VB) model was constructed and normally distributed populations for each age class of 1, 2, 3, and 4 yrs were simulated separately for each of two pigment classes, wet weight eyes and carapace length. Each of these predictor variables was grouped using 22 classes, and the frequency of animals in each variable class by each age class was determined for each variable separately from the simulated data. These frequency data were then modeled using a form of ordinal regression called a continuation-ratio logistic regression, and from the fit of this model the proportion in each age class by variable was recovered (Candy, 1991, 2003). To determine how well each variable predicted age class, the percentage deviance explained by the continuation-ratio logistic regressions (Candy, 1991) was calculated where this statistic is the ordinal regression analogue of the familiar  $R^2$  statistic.

## 5.4 Results

### *Sex ratio*

There was a sex bias in the reared krill population. Female krill dominated the population samples at each age class, with the sex ratio rising from 2:1 (F:M) for

12 months-old krill, to 5:1 for krill aged 36 and 48 months-old (Table 6). Because the krill were randomly sampled from the aquarium population for age analysis, rather than selected by sex, there is subsequently more data available for female krill. Due to smaller sample sizes, extra caution is applied in interpreting the data for the male krill.

| Age (months) | Number of krill |      | Sex ratio |
|--------------|-----------------|------|-----------|
|              | Female          | Male |           |
| 12           | 17              | 8    | 2:1       |
| 24           | -               | -    | -         |
| 36           | 10              | 2    | 5:1       |
| 48           | 10              | 2    | 5:1       |

**Table 6.** Sex ratio of an Antarctic krill population reared over 48 months in the laboratory.

### *Larval krill*

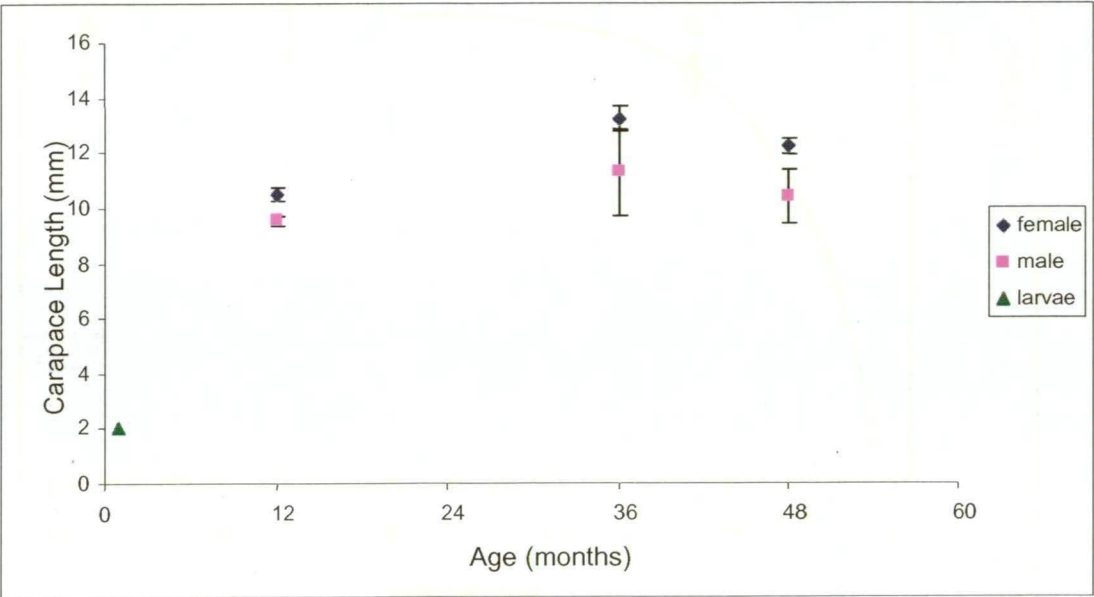
The larval krill, which were 1 month old, were at calyptopis stage (staged according to Kirkwood, (1982)). These krill were too small for precise morphometric measurement in the time-frame required for pigment extraction. This is because sample warming (which occurs quickly in such small animals) might influence pigment fluorescence. Therefore the larval krill were briefly assessed visually, and were estimated to average about 2 mm in total length, with the carapace constituting between half and two thirds of the total length. Therefore to facilitate analysis and modeling, it was conservatively assumed that the mean larval krill carapace length (CL) was 2mm, although in reality it was always slightly smaller than this.

### *Carapace length*

The mean carapace lengths of the known age krill were calculated and plotted for each age class (Figure 15). Female krill were significantly larger than male krill at 12 and 48 months old (Table 7,  $P < 0.05$ ). Female krill were also larger than male krill at 36 months old, however this was not statistically discernable. All krill exhibited a positive increase in size from 1 month old larvae to 36 month old adults (Figure 15). There was no significant difference in size for either sex

between 36 and 48 months old, although visually there appears to be a small decrease in mean carapace length between krill aged 36 and 48 months old (Figure 15).

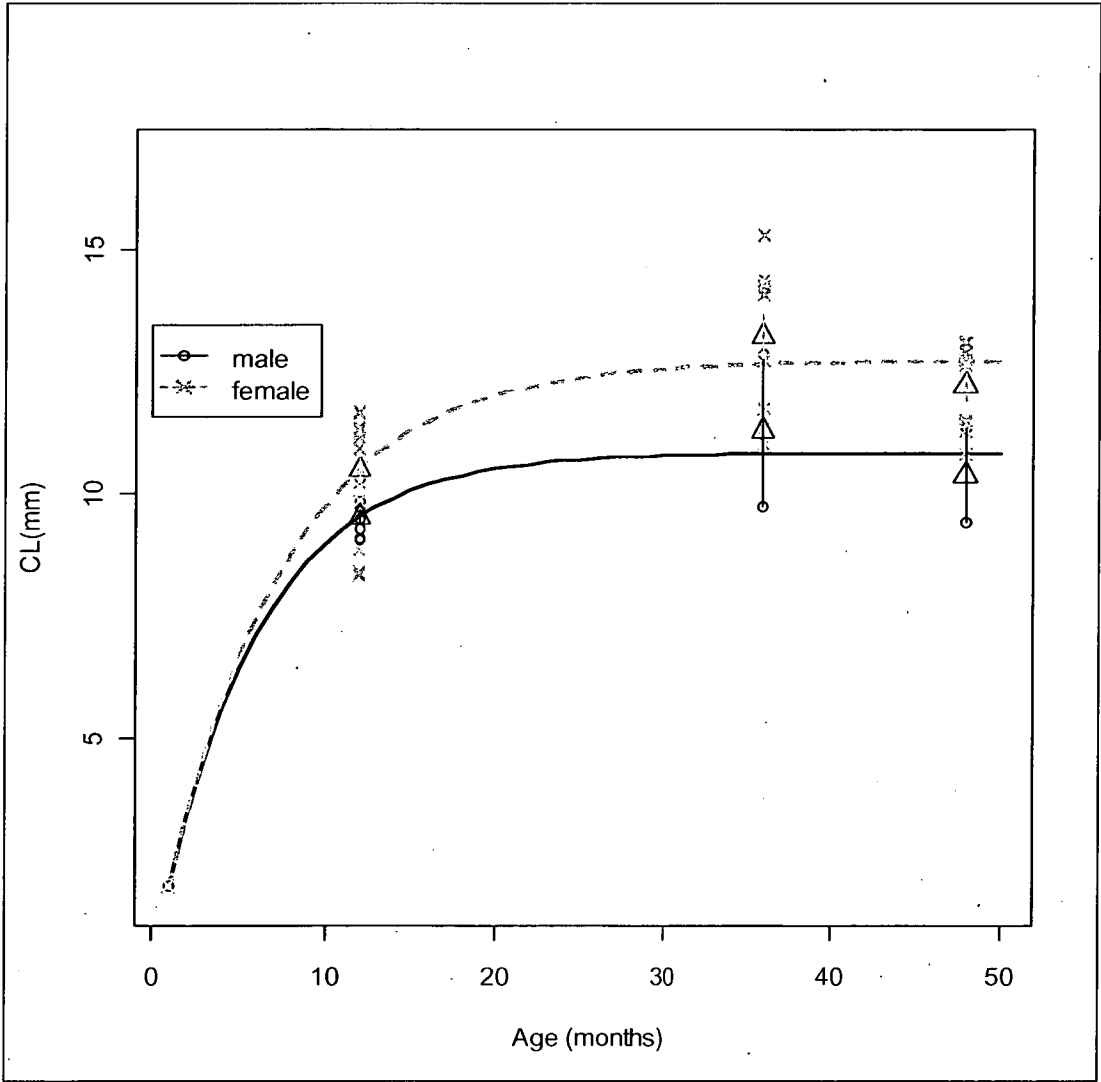
Carapace length (CL) at age fitted using a von Bertalanffy (VB) equation produced growth curves for the male and female krill (Figure 16). Two separate growth curves were produced for the male and female krill, because there was a statistically significant difference between their CLs at 12 and 48 months of age. The growth trajectory for the female krill was steeper, and the asymptote higher, than that for the male krill.



**Figure 15.** Mean carapace length (CL) of male, female and larval krill of known age. For males: n=8 at 12 mths, n= 2 at 36 mths and n=2 at 48 mths. For females n=17 at 12 mths, n=10 at 36 mths and n=10 at 48 mths. For larvae n=25.

| Age<br>(months) | CL (mm) ± SE |              |              | DF    | F<br>value | P<br>value | Signif.<br>(α = 0.05) |
|-----------------|--------------|--------------|--------------|-------|------------|------------|-----------------------|
|                 | Larvae       | Male         | Female       |       |            |            |                       |
| 1               | 2            |              |              |       |            |            |                       |
| 12              |              | 9.53 ± 0.14  | 10.51 ± 0.25 | 1, 23 | 6.1649     | 0.0207     | **                    |
| 36              |              | 11.30 ± 1.55 | 13.22 ± 0.46 | 1, 10 | 2.5768     | 0.1395     | NS                    |
| 48              |              | 10.38 ± 0.97 | 12.21± 0.26  | 1, 10 | 6.8236     | 0.0259     | **                    |

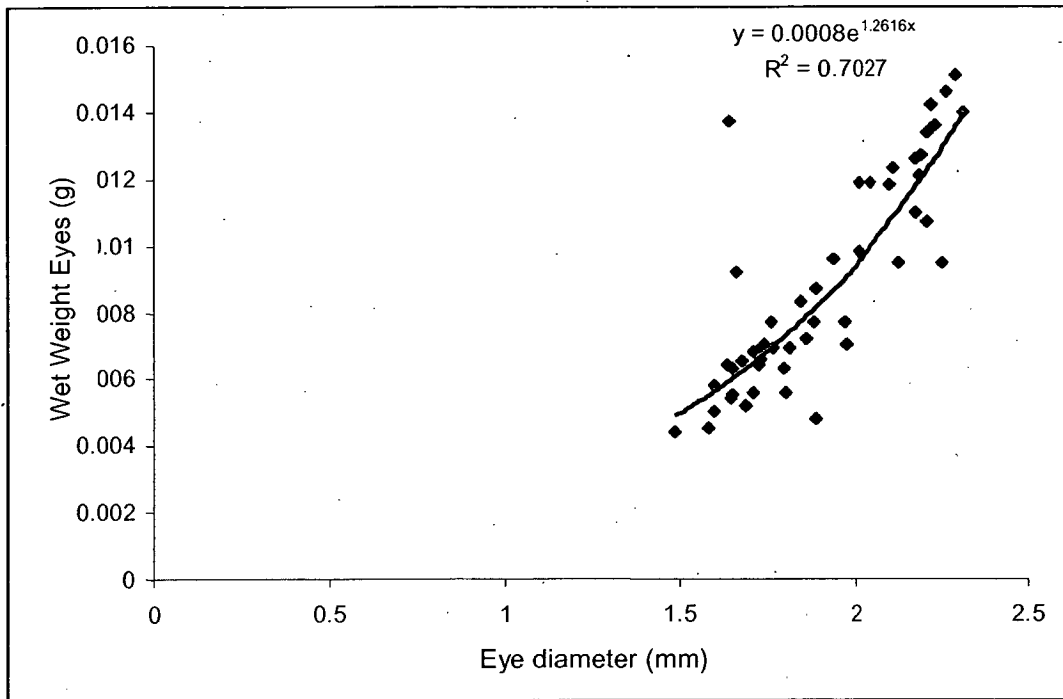
**Table 7.** Size of larval, male and female krill in each age class. Differences in means were analysed by one-way ANOVA. Significant differences are indicated by \*\*. The larval size is a conservative estimate of mean (see text) for 25 individuals.



**Figure 16.** Von Bertalanffy (VB) growth models fitted for carapace length of known-age male and female krill.

*Wet weight of eyes*

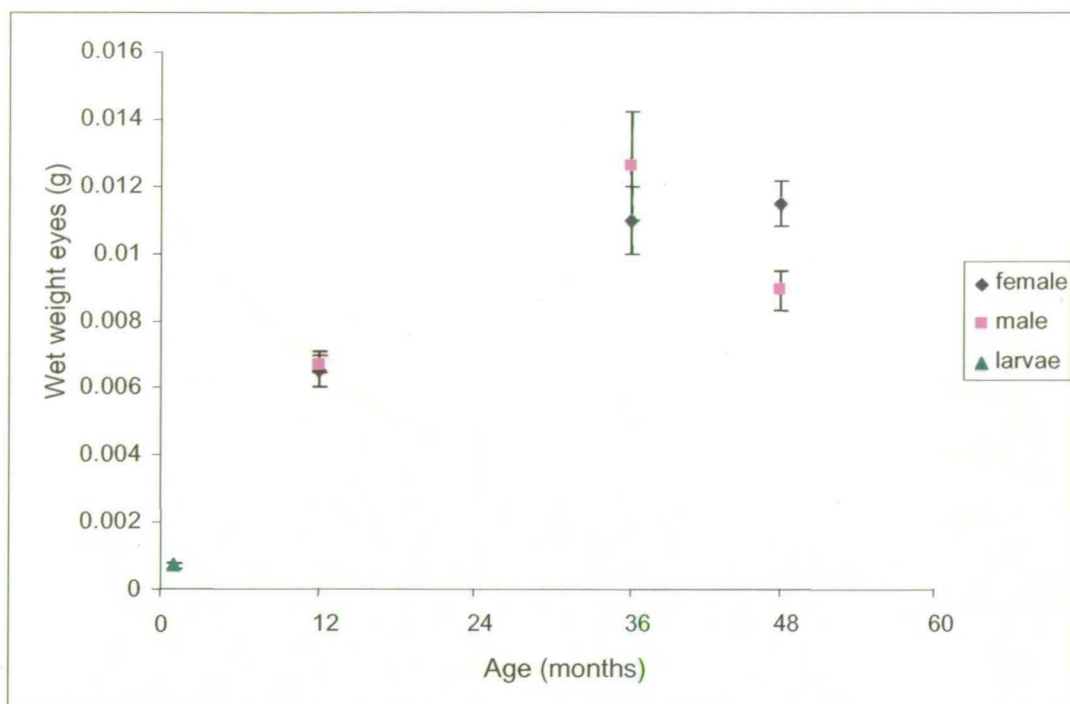
The eye diameter of the adult krill was plotted against the wet weight of dissected eyes plus eyestalks (Figure 17). The relationship was described by the exponential equation  $y = 0.0008e^{1.2616x}$  ( $r^2 = 0.7027$ ). The wet weight of the eyes (WWE) gave a more sensitive measure of increasing eyeball size than did eye diameter, and so this parameter was chosen for further analysis in relation to age.



**Figure 17.** Relationship between the wet weight of dissected eyes (plus eyestalks) and eye diameter of Antarctic krill.

The wet weight of the krill eyes was then plotted against age (Figure 18). There was a significant increase in eye weight with age up to three years, after which time there was no change in eyeball weight ( $DF_{3,47}$ ,  $F=26.06$ ,  $P<0.001$ ). There was no overall significant difference in the wet weight of the eyes related to sex (Table 8), and the data were pooled to fit a VB growth curve (Figure 19). The VB growth curve fitted on the wet weight of the eyes did not reach an asymptote by 48 months, indicating a better predictor of age than from the VB fit of carapace length.

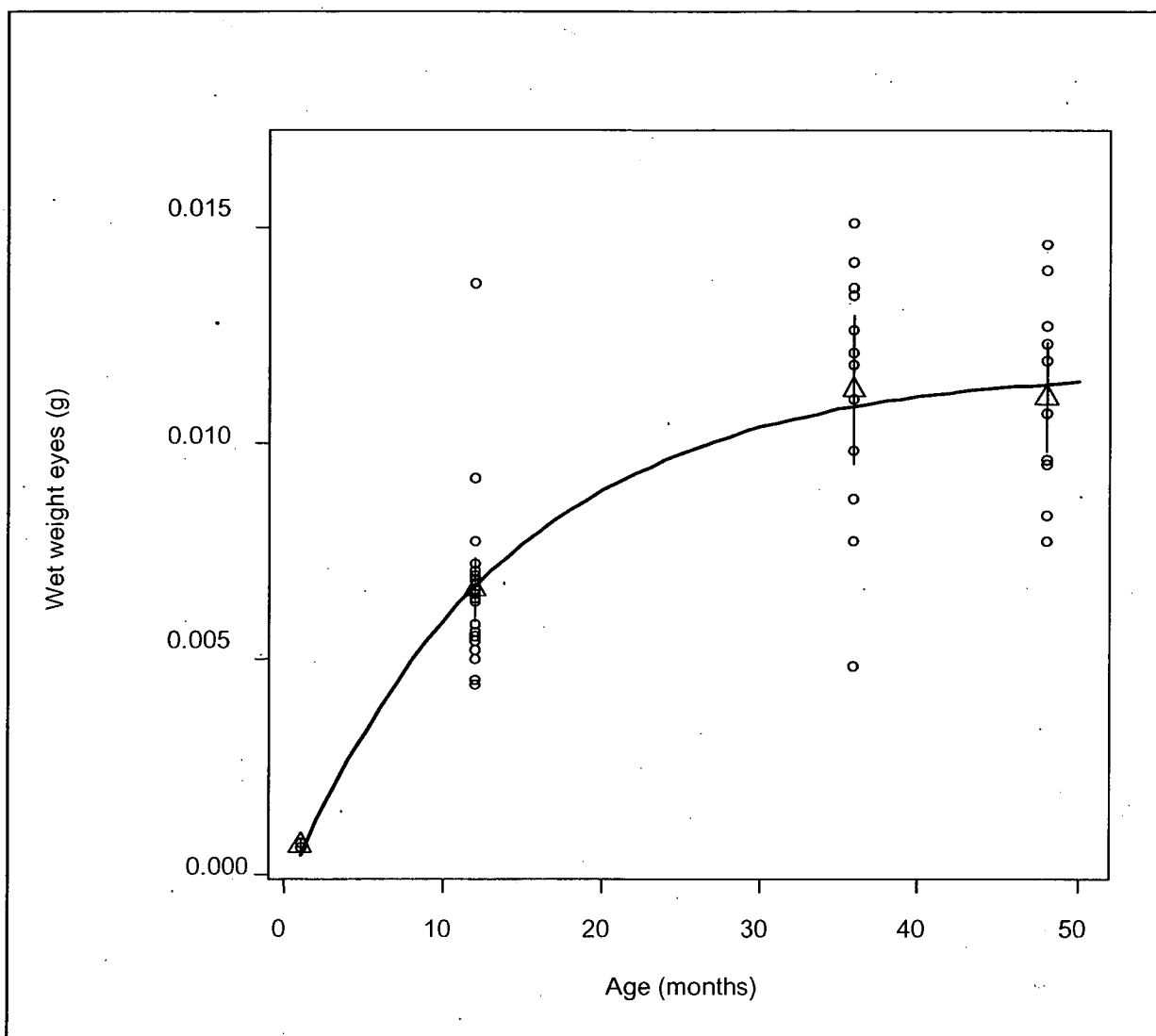




**Figure 18.** The wet weight of dissected eyes plus eyestalks (both) for female and male krill. The weight of whole larval krill is also shown. For males:  $n=8$  at 12 mths,  $n=2$  at 36 mths and  $n=2$  at 48 mths. For females  $n=17$  at 12 mths,  $n=10$  at 36 mths and  $n=10$  at 48 mths. For larvae  $n=25$ .

| Age<br>(months) | WWE (g) $\pm$ SE         |                           |                           | DF    | F<br>value | P<br>value | Significant<br>( $\alpha = 0.05$ ) |
|-----------------|--------------------------|---------------------------|---------------------------|-------|------------|------------|------------------------------------|
|                 | Larvae                   | Male                      | Female                    |       |            |            |                                    |
| 1               | 0.0007 $\pm$<br>7.66E-05 |                           |                           |       |            |            |                                    |
| 12              |                          | 0.0067<br>$\pm$<br>0.0003 | 0.0065<br>$\pm$<br>0.0005 | 1, 23 | 0.0500     | 0.8250     | NS                                 |
| 36              |                          | 0.0126<br>$\pm$<br>0.0016 | 0.0110<br>$\pm$<br>0.0010 | 1, 10 | 0.4723     | 0.5075     | NS                                 |
| 48              |                          | 0.0089<br>$\pm$<br>0.0006 | 0.0115<br>$\pm$<br>0.0007 | 1, 10 | 2.6831     | 0.1325     | NS                                 |

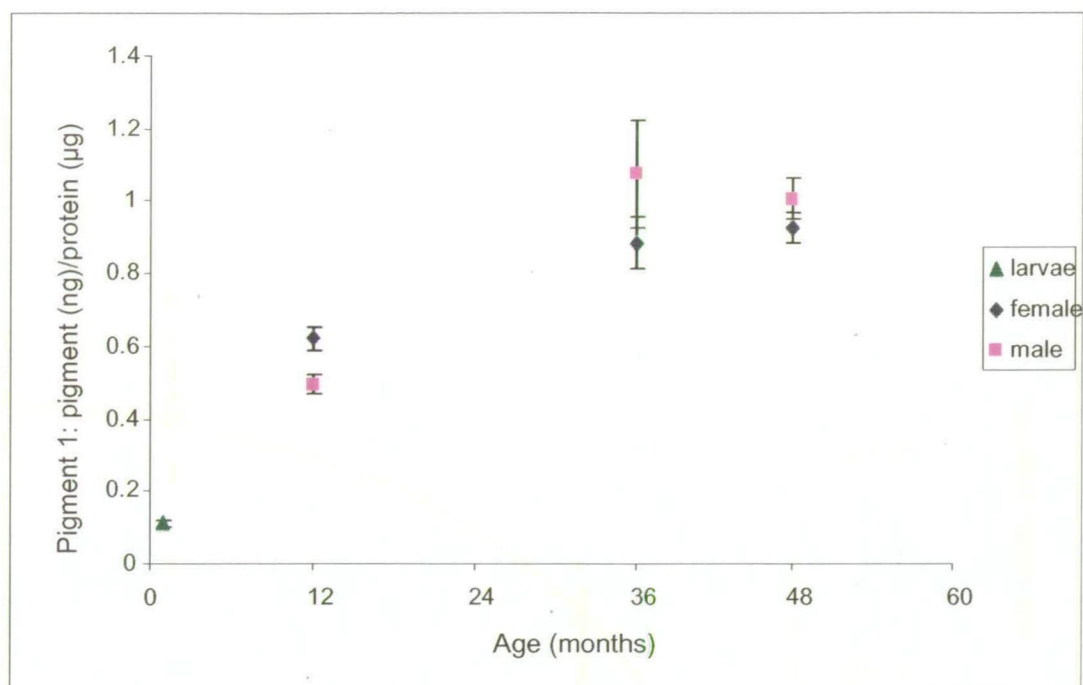
**Table 8.** Wet weight of dissected eyes of larval, male and female krill in each age class. Differences in means were analysed by one-way ANOVA. No significant differences (NS) were found between male and female krill ( $\alpha = 0.05$ ). The larval weight is the mean body weight of 25 whole individuals.



**Figure 19.** Von Bertalanffy (VB) growth model for wet weight eyes from known-age krill. Data for male and female krill were pooled.

### *Pigment 1*

The fluorescence peak intensities of extracted pigment measured at  $\lambda_{ex}=280\text{nm}$ ,  $\lambda_{em}=625\text{nm}$  were calibrated against a quinine sulphate standard, and then standardised against the amount of protein in each sample (see Chapter 3). The calculated amounts of pigment were then plotted against age for the larval, male and female krill (Figure 20). The mean quantity of pigment was significantly higher for female krill at age 12 months, but there was no difference attributable to sex at ages 36 or 48 months (Table 9).



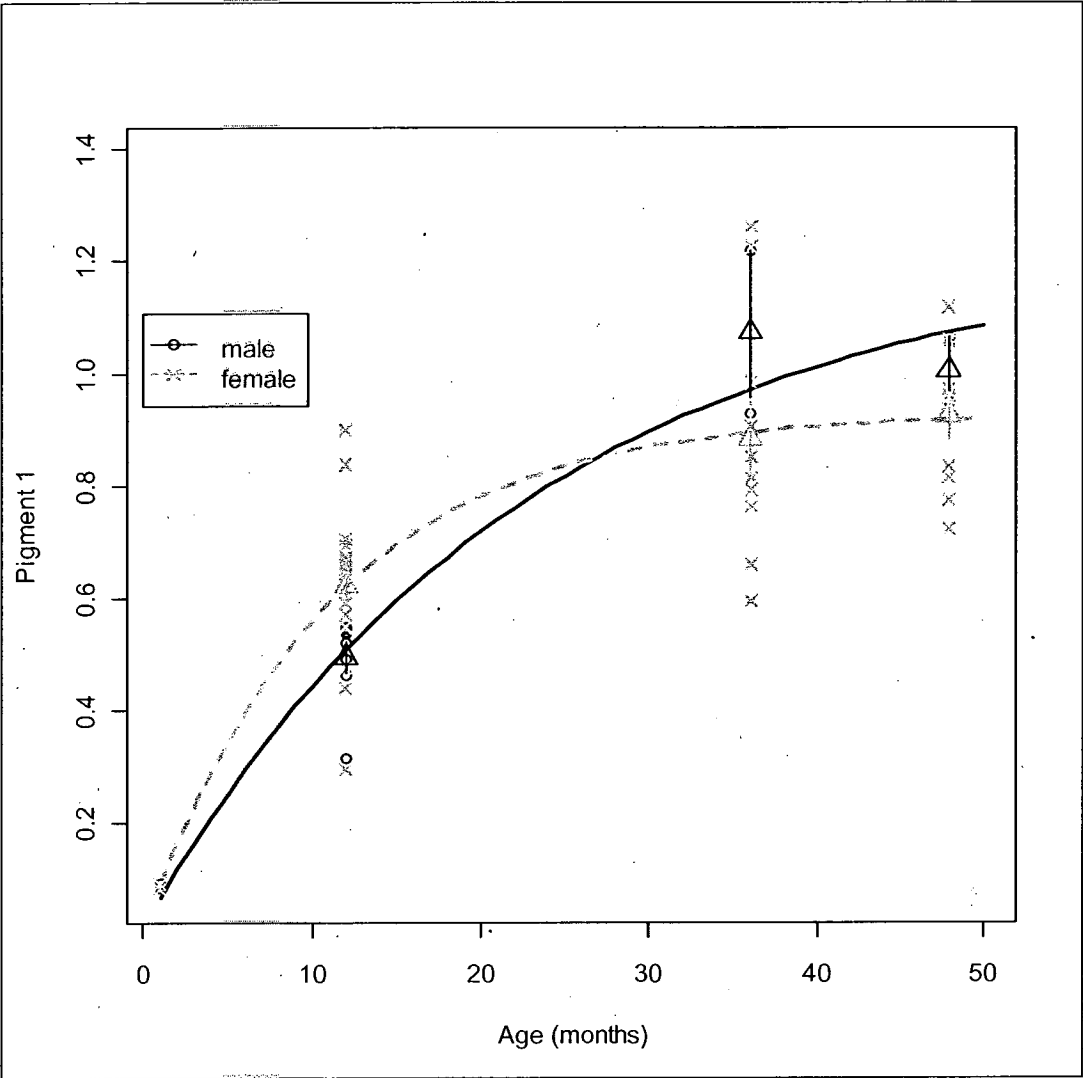
**Figure 20.** Quantity (mean  $\pm$  SE) of pigment 1 measured in larval, male and female krill of known-age. Fluorescence measured at  $\lambda_{ex}$ =280nm,  $\lambda_{em}$ =625nm and standardised against sample protein. For males: n=8 at 12 mths, n= 2 at 36 mths and n=2 at 48 mths. For females n=17 at 12 mths, n=10 at 36 mths and n=10 at 48 mths. For larvae n=25.

| Age<br>(months) | Pigment 1<br>(ng pigment/ug protein) $\pm$ SE |                           |                            | DF   | F<br>value | P<br>value | Significant<br>( $\alpha = 0.05$ ) |
|-----------------|-----------------------------------------------|---------------------------|----------------------------|------|------------|------------|------------------------------------|
|                 | Larvae                                        | Male                      | Female                     |      |            |            |                                    |
| 1               | 0.1101<br>$\pm$<br>0.0096                     |                           |                            |      |            |            |                                    |
| 12              |                                               | 0.4951<br>$\pm$<br>0.0278 | 0.6207<br>$\pm$<br>0.0342  | 1,23 | 5.4647     | 0.0285     | **                                 |
| 36              |                                               | 1.0732<br>$\pm$<br>0.1459 | 0.8831<br>$\pm$<br>0.0693  | 1,10 | 1.2697     | 0.2861     | NS                                 |
| 48              |                                               | 1.0047<br>$\pm$<br>0.0585 | 0.9236<br>$\pm$<br>0.04226 | 1,10 | 0.6551     | 0.4371     | NS                                 |

**Table 9.** Quantity of pigment 1 in larval, male and female krill in each age class. Differences in means were analysed by one-way ANOVA. Significant difference is indicated by \*\*. No significant difference is indicated by NS ( $\alpha = 0.05$ ).

There was a highly significant increase in pigment 1 with age for the female krill ( $DF_{3,35}$ ,  $F=20.2026$ ,  $P<0.001$ ) and the male krill ( $DF_{3,10}$ ,  $F= 48.1631$ ,  $P<0.001$ ). Because a difference in quantity of pigment related to sex was detected at one age class, the VB growth curves based on pigment 1 were fitted separately for each sex (Figure 21). Initially, quantity of pigment increases more quickly for

female krill, but after 24 months of age the accumulation rate drops for female krill, and is overtaken by the accumulation rate for male krill (Figure 21).

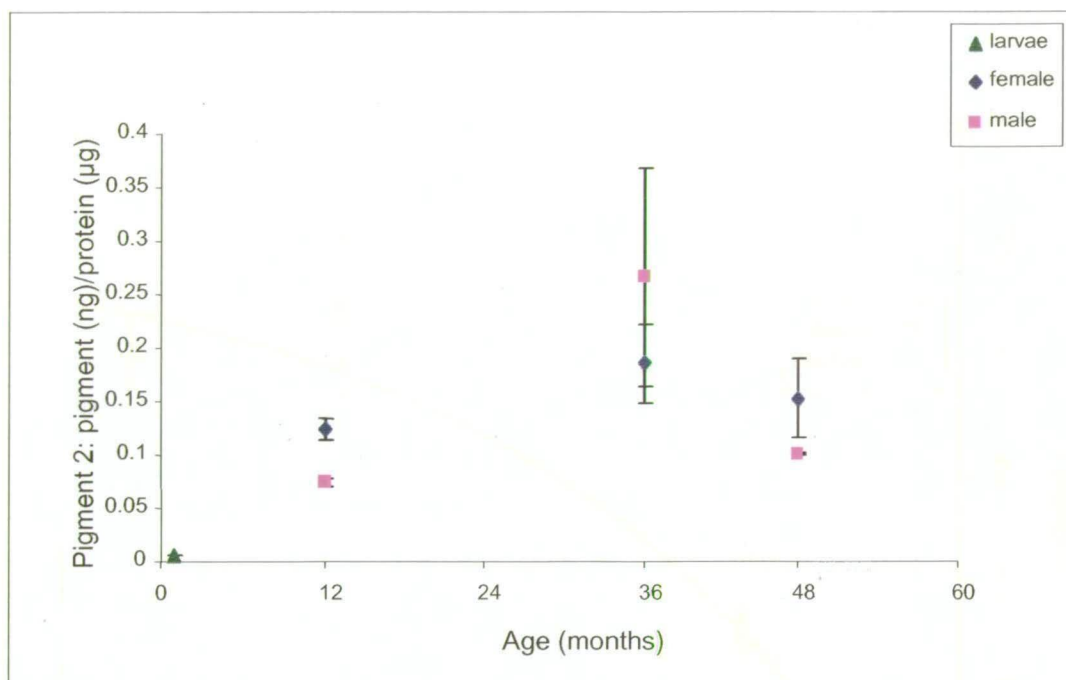


**Figure 21.** Von Bertalanffy growth models of pigment 1 with age in male and female known-age krill.

### *Pigment 2*

The calculated amounts of extracted pigment measured at  $\lambda_{ex}=355\text{nm}$ ,  $\lambda_{em}=510\text{nm}$  (pigment 2) were plotted against age for the larval, male and female krill (Figure 22). As for pigment 1, the 12 month-old female krill contained significantly more pigment 2 compared to males, but there was no statistical difference between male and female krill at 36 or 48 months of age (Table 10). Between 36 and 48 months of age the amount of pigment 2 decreased for both sexes, although the decrease

was not statistically discernable ( $DF_{1,22}$ ,  $F= 1.3921$   $P= 0.2507$ ). Pigment 2 was therefore discounted as an age-pigment, and the accumulation was not modelled.



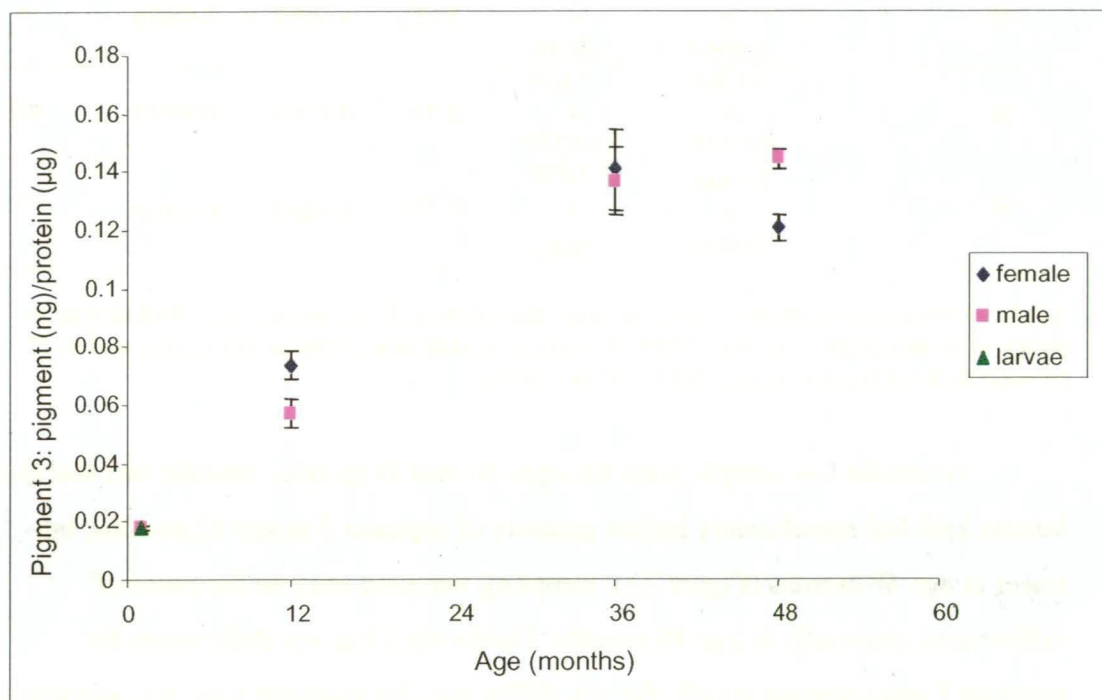
**Figure 22.** Quantity (mean  $\pm$  SE) of pigment 2 measured in larval, male and female krill of known-age. Fluorescence measured at  $\lambda_{ex}=355\text{nm}$ ,  $\lambda_{em}=510\text{nm}$  and standardised against sample protein. For males:  $n=8$  at 12 mths,  $n= 2$  at 36 mths and  $n=2$  at 48 mths. For females  $n=17$  at 12 mths,  $n=10$  at 36 mths and  $n=10$  at 48 mths. For larvae  $n=25$ .

| Age<br>(months) | Pigment 2<br>(ng pigment/ug protein) $\pm$ SE |                           |                           | DF   | F value | P<br>value | Significant<br>( $\alpha = 0.05$ ) |
|-----------------|-----------------------------------------------|---------------------------|---------------------------|------|---------|------------|------------------------------------|
|                 | Larvae                                        | Male                      | Female                    |      |         |            |                                    |
| 1               | 0.0060<br>$\pm$<br>0.0008                     |                           |                           |      |         |            |                                    |
| 12              |                                               | 0.0739<br>$\pm$<br>0.0049 | 0.1236<br>$\pm$<br>0.0098 | 1,23 | 11.1949 | 0.0028     | **                                 |
| 36              |                                               | 0.2659<br>$\pm$<br>0.1028 | 0.1856<br>$\pm$<br>0.0370 | 1,10 | 0.7427  | 0.4090     | NS                                 |
| 48              |                                               | 0.1009<br>$\pm$<br>0.0005 | 0.1529<br>$\pm$<br>0.0372 | 1,10 | 0.3608  | 0.5614     | NS                                 |

**Table 10.** Quantity of pigment 2 in larval, male and female krill in each age class. Differences in means were analysed by one-way ANOVA. Significant difference is indicated by \*\*. No significant difference is indicated by NS ( $\alpha = 0.05$ ).

## Pigment 3

The calculated amounts of extracted pigment measured at  $\lambda_{ex}=463$  nm and  $\lambda_{em}=620$  nm (pigment 3) were plotted against age for the larval, male and female krill (Figure 23). There was a significantly higher quantity of pigment 3 in the female krill compared to the males at 12 months of age (Table 11).



**Figure 23.** Quantity (mean  $\pm$  SE) of pigment 3 measured in larval, male and female krill of known-age. Fluorescence was measured at  $\lambda_{ex}=463$  nm and  $\lambda_{em}=620$  nm and standardised against sample protein. For males:  $n=8$  at 12 mths,  $n=2$  at 36 mths and  $n=2$  at 48 mths. For females  $n=17$  at 12 mths,  $n=10$  at 36 mths and  $n=10$  at 48 mths. For larvae  $n=25$ .

This sex-related difference at 12 months was also the case for pigments 1 and 2. There was also a significantly higher quantity of pigment 3 in male krill compared to females at 48 months of age (Table 11). There were significant differences in the quantity of pigment 3 attributable to sex, therefore the male and female krill were initially analysed separately. There was a significant increase in pigment with age for the female krill ( $DF_{3,35}$ ,  $F=21.3850$ ,  $P<0.001$ ) and the male krill ( $DF_{3,10}$ ,  $F=53.9326$ ,  $P<0.001$ ). Further analysis calculated a pooled Residual Mean Square (RMS) by combining the sex data for each year. The RMS had 43 degrees of freedom so it was more powerful, in terms of giving a better estimate of between-

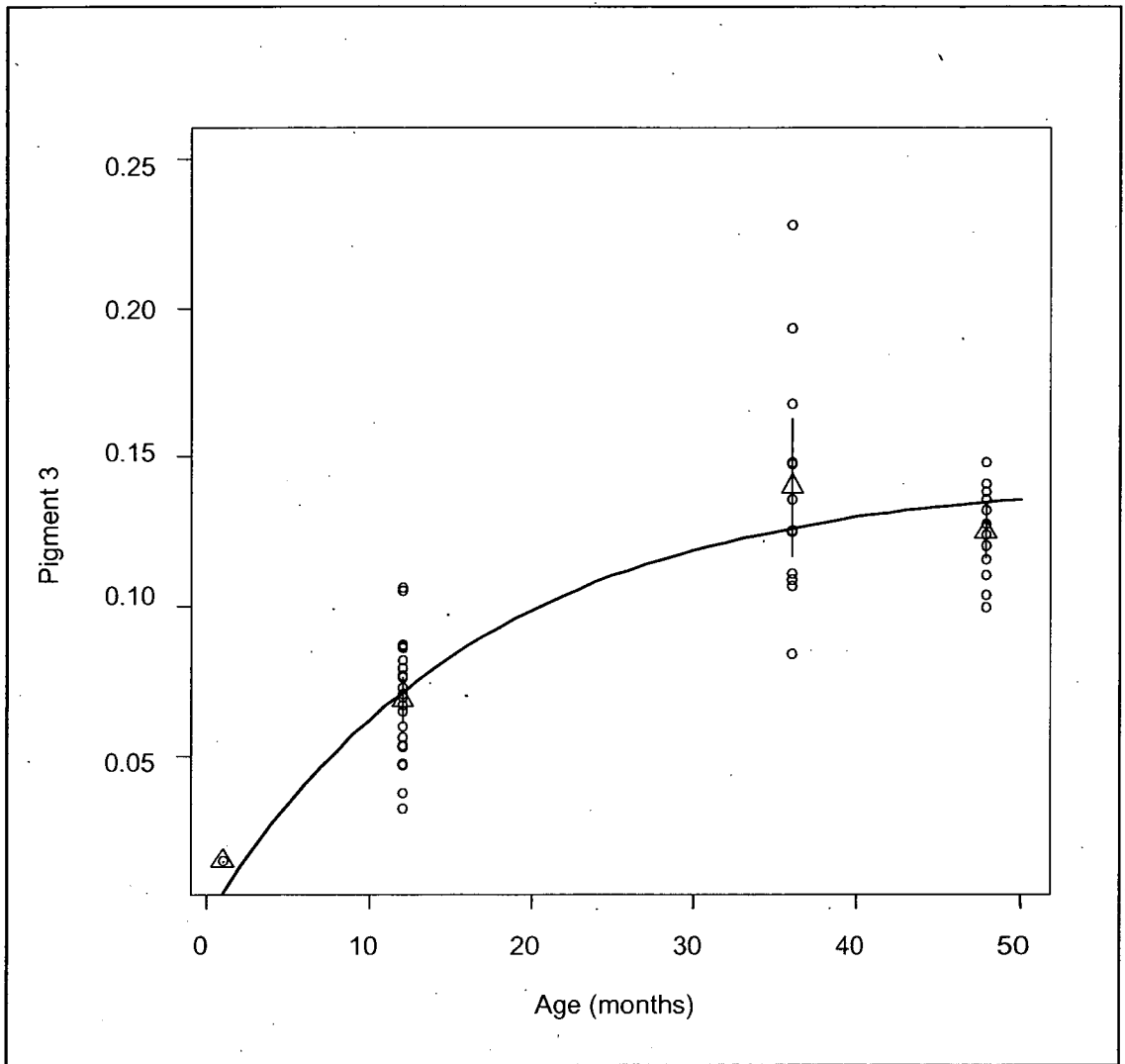
animal variance than the individual ANOVAs, given the assumption that the variance is the same for each age.

| Age<br>(months) | Pigment 3<br>(ng pigment/ $\mu$ g protein) $\pm$ SE |                           |                            | DF   | F value | P value | Significant<br>( $\alpha = 0.05$ ) |
|-----------------|-----------------------------------------------------|---------------------------|----------------------------|------|---------|---------|------------------------------------|
|                 | Larvae                                              | Male                      | Female                     |      |         |         |                                    |
| 1               | 0.0182<br>$\pm$<br>0.0008                           |                           |                            |      |         |         |                                    |
| 12              |                                                     | 0.0570<br>$\pm$<br>0.0049 | 0.0739<br>$\pm$<br>0.0046  | 1,23 | 4.9656  | 0.0359  | **                                 |
| 36              |                                                     | 0.1365<br>$\pm$<br>0.0116 | 0.1407<br>$\pm$<br>0.01310 | 1,10 | 0.0163  | 0.9009  | NS                                 |
| 48              |                                                     | 0.1444<br>$\pm$<br>0.0035 | 0.1208<br>$\pm$<br>0.0042  | 1,10 | 5.7847  | 0.0370  | **                                 |

**Table 11.** Quantity of pigment 3 in larval, male and female krill in each age class. Differences in means were analysed by one-way ANOVA. Significant difference between sex is indicated by \*\*. No significant difference is indicated by NS ( $\alpha = 0.05$ ).

Given the low sample sizes for ages 36 and 48 months, and the fact that the female krill had significantly higher quantity of pigment 3 at age 12 months, but lower at age 48 months (Figure 23), sampling variation may be the cause of differences, especially at age 48 months. Unlike the clear sex differences for pigment 1 and carapace length, the sex differences for pigment 3 are less apparent, and more power is brought to the analysis by combining the data. The sex data were pooled, therefore, to produce the VB growth curve for pigment 3 (Figure 24).





**Figure 24.** Von Bertalanffy growth model of pigment 3 with age in known-age krill.

#### *Testing the fit of the relationships with a simulated population*

Normally distributed populations for each age class of 12, 24, 36, and 48 months were simulated by modeling the mean and variance in the real data for the known-age krill (obtained from the fit of the von Bertalanffy model for each, above; Appendix 2). Carapace length, wet weight of eyes and pigment 3 were modeled, using 1000 samples with each variable grouped into 22 classes. Continuation ratio regressions were fitted for each variable and age, and the proportion in each age for each class of the variable was recovered. These are shown plotted as variable (CL, WWE, Pigment 3) against predicted proportion of the population (Figures 25-27). These figures were examined to identify separation in the bell curves for ages



2 and 3 years in the middle, and the sigmoidal curves at each end for ages 1 and 4 years. The percent deviance explained provides a good measure of this separation (Table 12).

Carapace length had the weakest relationship with age. There was poor separation of the curves based on carapace length for either the female or male krill (Figures 25 & 26). This is reflected by the percent deviance explained of 8.3% and 14.4%, respectively (Table 12).

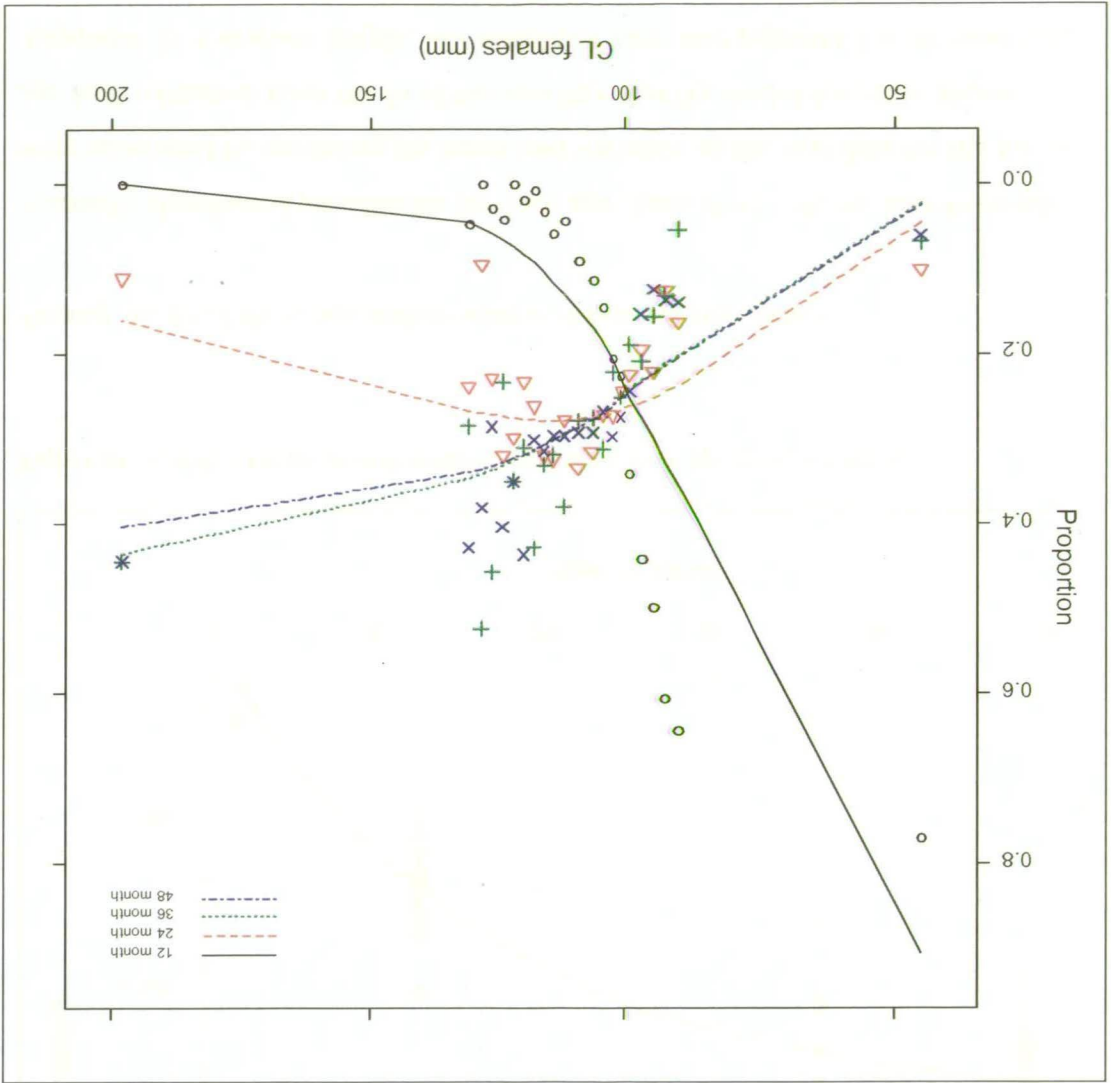
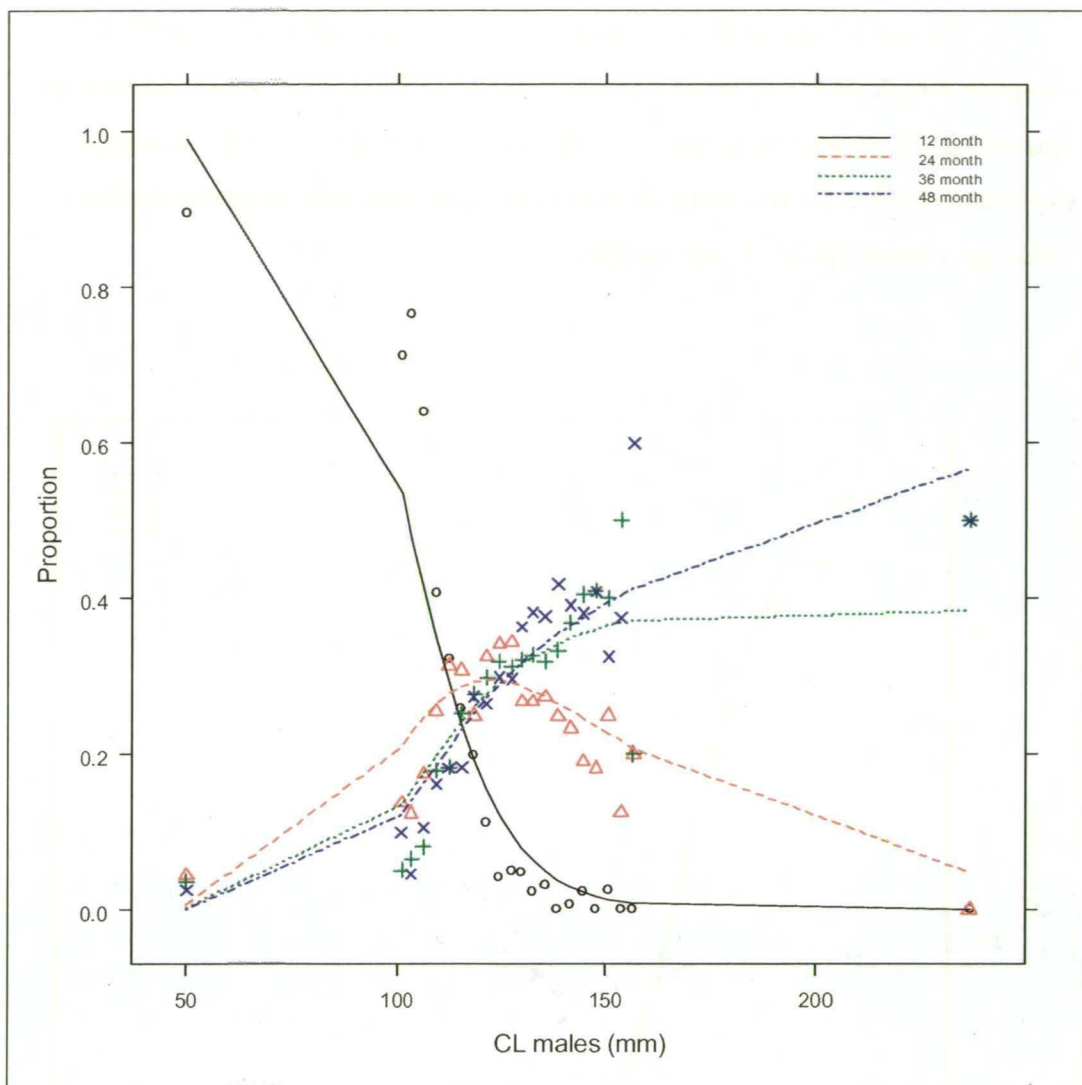


Figure 25. Continuation regression of carapace length (CL) of female krill with simulated proportion in population at 12, 24, 36 and 48 months of age.

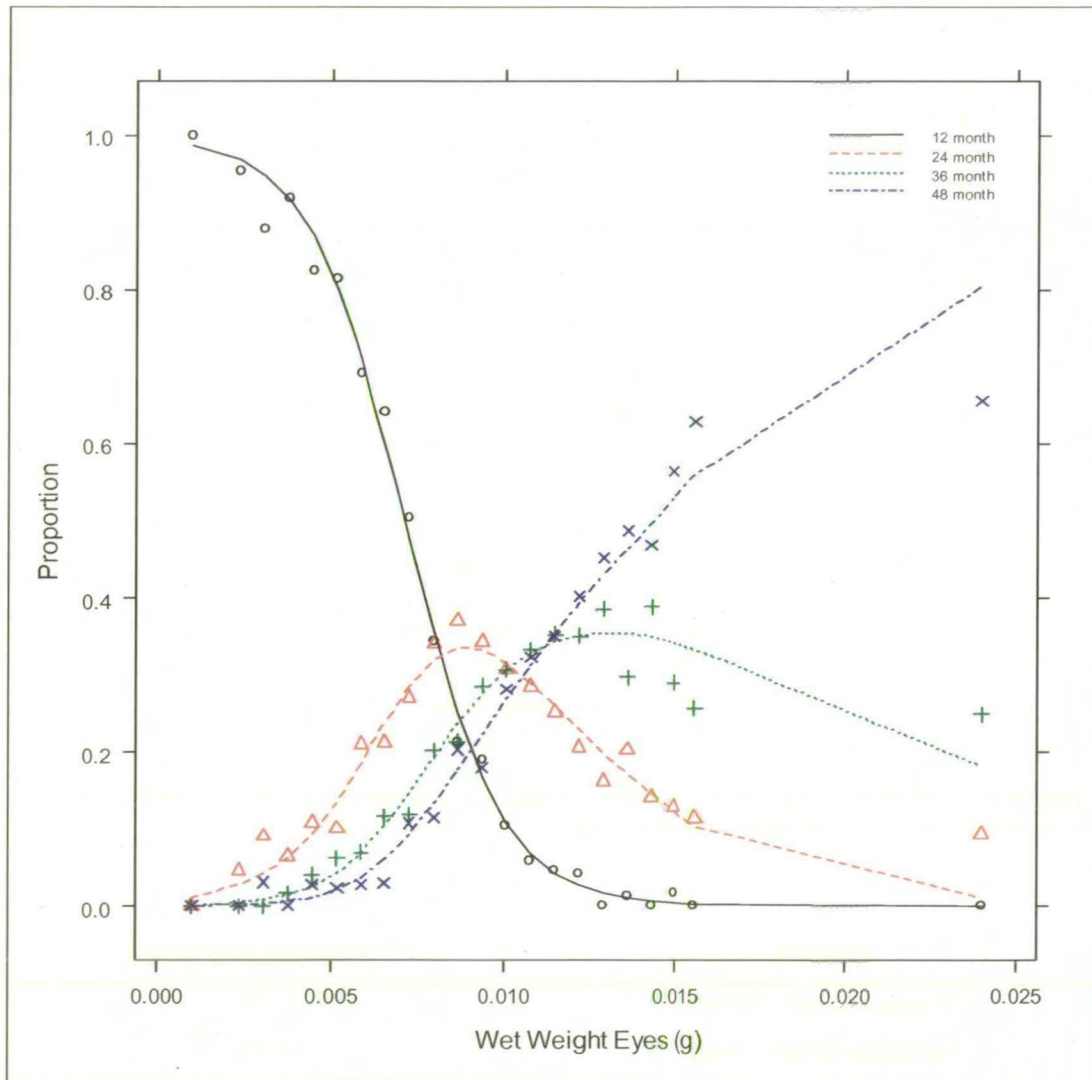


**Figure 26.** Continuation regression of carapace length (CL) of male krill with simulated proportion in population at 12, 24, 36 and 48 months of age.

| Variable                    | % Deviance explained |
|-----------------------------|----------------------|
| Carapace length (female)    | 8.3%                 |
| Carapace length (male)      | 14.4%                |
| Wet weight eyes             | 18.5%                |
| Pigment 3                   | 21.4%                |
| Pigment 3 (with reduced SD) | 63.4%                |

**Table 12.** Fit of the relationship of each variable examined with age. The ‘% deviance explained’ describes the separation of normal population curves for krill aged 12, 24, 36 and 48 months of age based on each variable, and is analogous to the familiar  $R^2$  statistic.

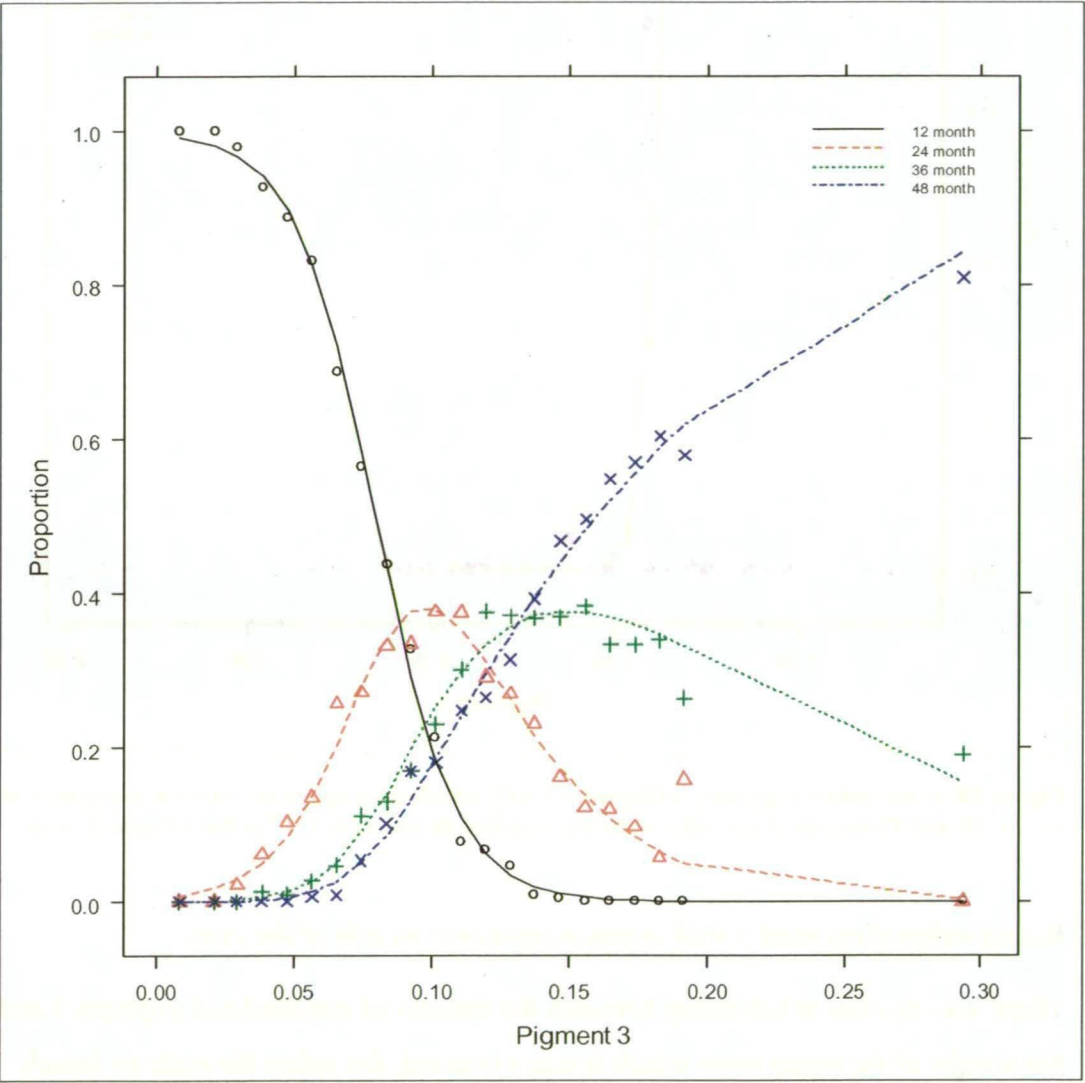
The wet weight of the eyes had a stronger relationship with age than carapace length. There is better separation between the normal curves for the age classes 12, 24, 36 and 48 months old (Figure 27), with 18.5% of the deviance explained (Table 12). It is difficult, however, to discriminate between the three older age classes based on wet weight eyes.



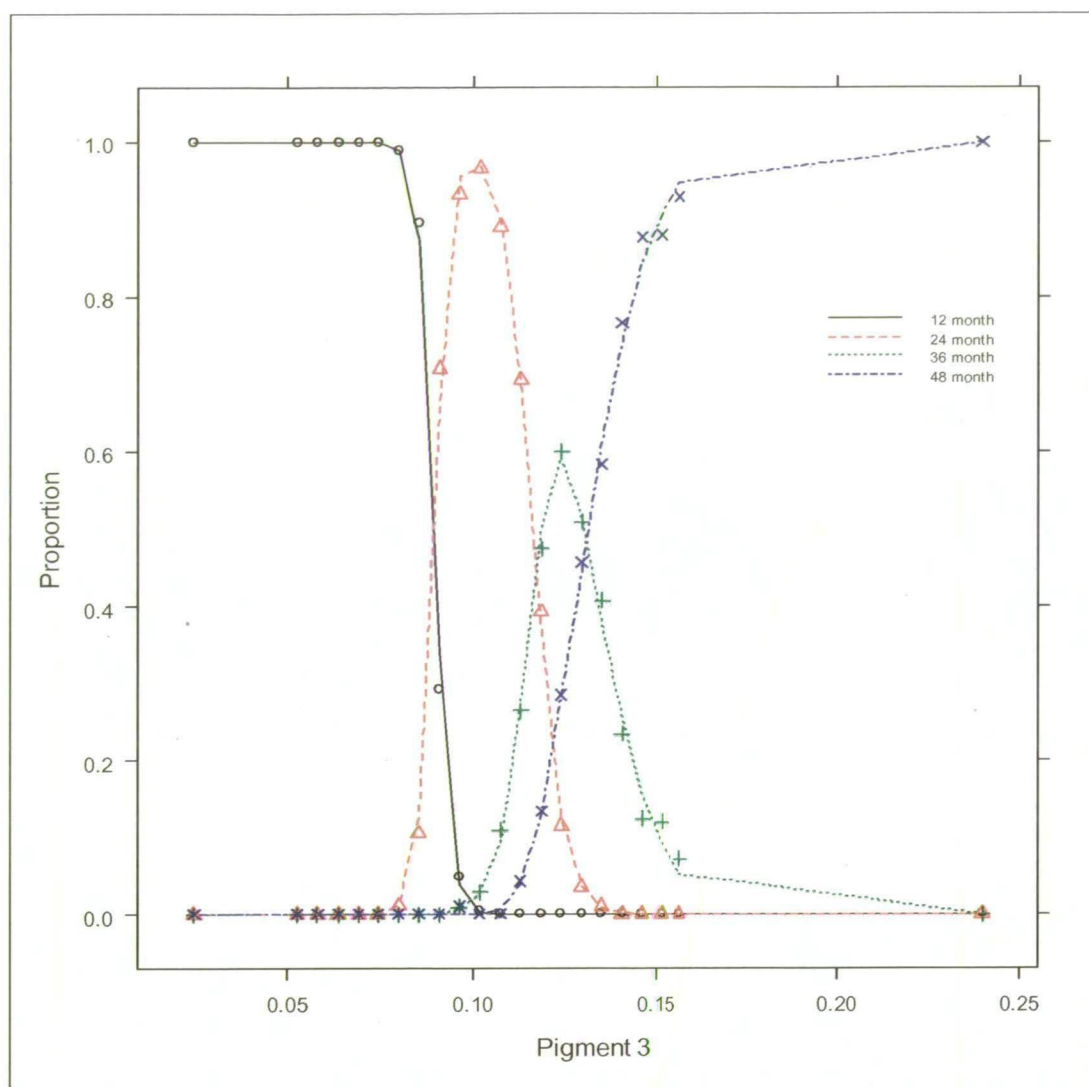
**Figure 27.** Continuation regression of wet weight eyes of krill with simulated proportion in population at 12, 24, 36 and 48 months of age.

Pigment 3 had the strongest relationship with age. Separation of the distribution curves for each age based on pigment 3 was clearer than for carapace length or wet weight of the eyes (Figure 28). The deviance explained was 21.4% (Table 12). Despite Pigment 3 having the strongest correlation with age, it is difficult to discriminate between ages 24, 36, and 48 months old on this basis, due to the high

variance (Figure 28). The continuation regression of pigment 3 was then remodelled with the variance reduced to a third of its original value (Figure 29). This was done to evaluate if pigment 3 would be a more robust predictor of age if the variance could be methodologically reduced. The deviance explained rose to 63.4% by reducing the variance (Table 12).



**Figure 28.** Continuation regression of pigment 3 with simulated proportion krill of in population at 12, 24, 36 and 48 months of age.

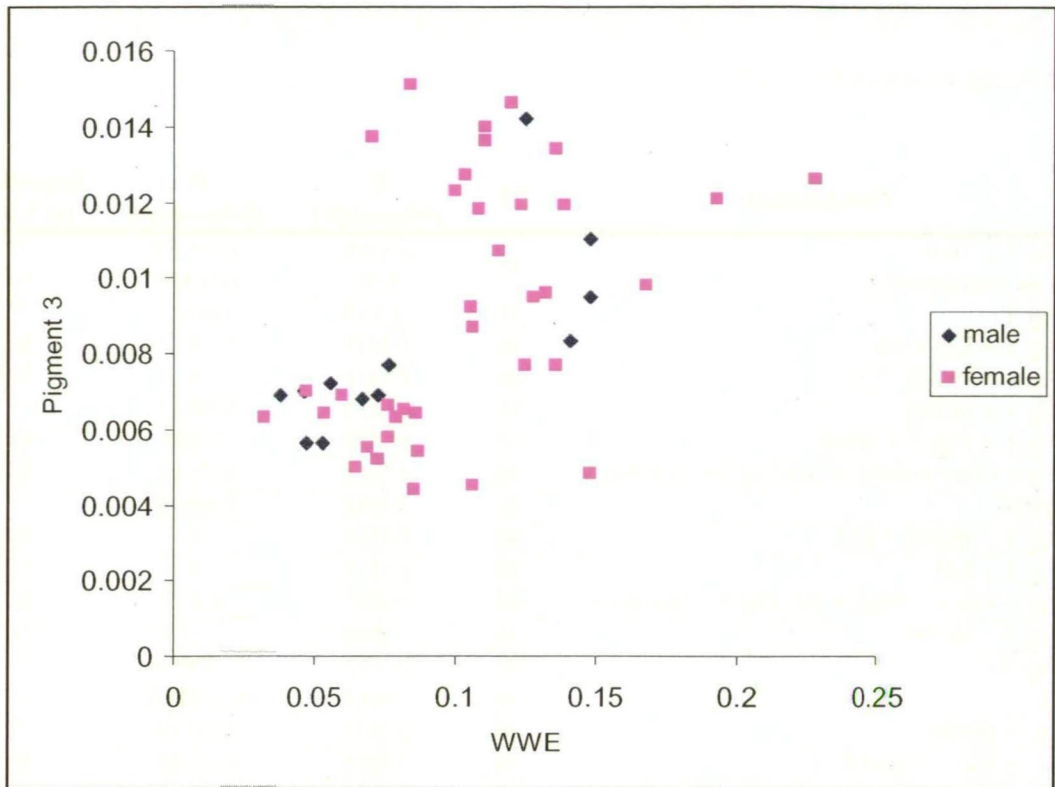


**Figure 28.** Continuation regression of pigment 3 with simulated proportion of krill in population at 12, 24, 36 and 48 months of age, after reducing standard deviation to  $1/3^{\text{rd}}$  of the estimated value.

#### *Relationship of pigment 3 with organ weight (wet weight of the eyes)*

There was no clear relationship between the amount of standardised pigment 3 and the weight of the organ from which it was extracted, for either the male or female krill (wet weight of the eyes plus eyestalks: WWE, Figure 29).





**Figure 29.** Pigment 3 plotted against wet weight of eyes (WWE) for the male and female krill aged 12, 36 and 48 months.

*Logistic discriminant analysis: Predicting age from carapace length, wet weight of the eyes and extracted pigment*

The variables of carapace length (CL), sex, eye diameter (ED), wet weight of the eyes (WWE), pigment 1 and pigment 3 were analysed for a relationship with age, using a linear model in the software package R (R Development Core Team, 2008; Appendix 3). Pigment 1 (Fig.1), pigment 3 (Fig.3), CL, WWE, pigment 1 + WWE, pigment 3 + WWE and CL + WWE were found to be significantly related to age (Table 13).

The variables that significantly correlated with age were then used for a logistic discriminant analysis, to assess the ability of each component to correctly sort the krill aged 12 and 36 months old (Appendix 3). Carapace length and pigment 3 correctly discriminated 100% of the 12 month old krill (Table 14). For the 36 month old krill, however, pigment 1 + WWE and pigment 3 + WWE predicted age correctly 100% of the time (Table 14). Overall, pigment 3 combined

with wet weight of the eyes provided the best predictor of age. There was no detectable interaction with sex.

| Component                               | DF | Z<br>(Intercept) | P<br>(Intercept) | Significant<br>( $\alpha = 0.05$ ) |
|-----------------------------------------|----|------------------|------------------|------------------------------------|
| Pig.1 + Sex<br>(sex component)          | 36 | 2.544<br>0.06    | 0.0110<br>0.9949 | **<br>NS                           |
| Pig.1                                   | 36 | 3.144            | 0.001            | **                                 |
| Pig.1 + ED + CL                         | 36 | 0.001            | 1                | NS                                 |
| Pig.1 + WWE + CL                        | 36 | 0.001            | 1                | NS                                 |
| Pig.1 + WWE                             | 36 | 2.023            | 0.0431           | **                                 |
| Pig.1 + Pig.3 + WWE                     | 36 | 0.008            | 0.994            | NS                                 |
| Pig.1 + Sex + WWE + Sex.Pig.1 + Sex.WWE | 36 | 1.925            | 0.0543           | NS                                 |
| WWE                                     | 36 | 3.592            | 0.0004           | **                                 |
| Pig.1 + WWE + ED                        | 36 | 0.001            | 1                | NS                                 |
| Pig.1 + ED                              | 36 | 0.001            | 1                | NS                                 |
| Pig.3 + Sex + WWE + Sex.Pig.3 + Sex.WWE | 36 | 0.887            | 0.375            | NS                                 |
| Pig.3 + WWE                             | 36 | 0.888            | 0.375            | NS                                 |
| Pig.3                                   | 36 | 2.702            | 0.00690          | **                                 |
| CL                                      | 36 | 2.843            | 0.00446          | **                                 |
| CL + WWE                                | 36 | 2.531            | 0.0114           | **                                 |
| CL + Sex + Sex.CL                       | 36 | 1.924            | 0.0544           | NS                                 |
| CL + Sex.CL + Sex + Pig.1 + Sex.Pig.1   | 36 | 0.001            | 1                | NS                                 |

**Table 13.** Results of a multivariate analysis of the relationship of carapace length, eye diameter, wet weight of the eyes, pigment 1, pigment 3 and sex (components and interactions) with age. Significant components and interactions are indicated by \*\*. Non-significant interactions are indicated by NS.

| Component   | Correctly predict age=1 y.o. | Correctly predict age=3 y.o. |
|-------------|------------------------------|------------------------------|
| CL          | 100%                         | 75%                          |
| Pig.1 + WWE | 92%                          | 100%                         |
| Pig.1       | 92%                          | 83%                          |
| Pig.3 + WWE | 96%                          | 100%                         |
| Pig.3       | 100%                         | 92%                          |

**Table 14.** Percentage of krill correctly placed in each age class by variable (component).

## 5.5 Discussion

### *Sex ratio*

The observed sex ratio in the reared krill population was female biased at all age classes, increasing to 5:1 by 36 months of age. This may be the result of sampling; however with a sample size of 49 krill, it is unlikely that this trend would have been observed at each age class unless it was representative of the population from

which the krill were sampled. This is further supported by finding the same sex ratio at each of the older age classes of 36 and 48 months.

Assuming equal numbers of male and female krill hatch, the female krill may have better survivorship than the male krill. Male krill may adopt a 'live fast, die young' strategy to optimise reproductive success (Kawaguchi *et al.*, 2007). The male krill may also be more susceptible to physiological stress. If male krill are dying at a higher rate than female krill, this would lead to a population sex bias that increases over time. This was observed up until age 36 months, after which time the sex bias remained constant, suggesting that another factor may be involved.

A more speculative explanation for this finding is that krill may be protandrous hermaphrodites. Many crustaceans have a life history strategy that involves sex change, and in many cases begin life as a male, and then change to female following cues from the population that would favour females for reproduction (Chiba, 2007). Protandrous hermaphroditism is thought to optimise reproductive success in the species that exhibit this trait. This has not been studied in krill and does not appear to have been considered thus far. That protandrous hermaphroditism may occur in Antarctic krill is speculative; however, if it does occur, information about krill biology may need revision. This suggests an interesting avenue for future research.

### *Larval krill*

The larval krill, which were 1 month old, were very small at ~2mm total length. This study shows that age-pigments are present in krill of this age and size in extractable quantities. The larval krill were pooled into groups of 12 and 13 for analysis to increase the chances of pigment detection. The technique was sensitive enough to quantify age-pigment for these size groups, and the number of krill in a pooled sample could be reduced. For small population samples this would increase the robustness of statistical analysis and modeling. Age-pigment has also been detected using a similar method in the krill *E. pacifica* at 3 months of age, when this species of krill is ~8 mm long (Harvey *et al.*, in press). The lower limits of detection were not established in the present study.



There is no practical need to age larval krill, since the developmental stage in itself provides an indication of age. The weight, estimated length and pigment quantities for the larval krill provided useful data for modeling growth and the accumulation of the pigments.

### *Carapace length*

Length analysis is the most widely used tool for age estimation of krill (Nicol, 2000). In this study the relationship of carapace length with age was investigated and compared with age-pigment and eye size. Several important limitations became apparent using carapace length to age krill. Firstly, the adult female krill had a greater carapace length than the male krill of the same age. This is part of normal sexual dimorphism in Antarctic krill (Reid & Measures, 1998), and is related to secondary morphological reproductive characteristics (ie fat reserves and egg production in females). The male and female krill must therefore be treated separately when using carapace length to predict other morphological features (eg total length) or to predict age (Goebel *et al.*, 2007). It is not always possible to sex krill, which regress to a sexually immature form under sub-optimal feeding conditions (McWhinnie *et al.*, 1979; Ikeda & Dixon, 1982). Lack of discrimination based on sex may therefore increase variance in length data, and confound attempts to use length to predict age.

Secondly, positive growth was not recorded between 36 months and 48 months of age, indicating that body size of adult krill is not a good indicator of age at these age classes. If krill live for 6-8 years, but do not significantly increase in size after 36 months of age, as suggested by the fit of the von Bertalanffy (VB) growth model, size must be used very cautiously to age krill older than 3 years of age. If this was occurring in wild krill there would be appear to be only three year classes, albeit with a disproportionate percentage of the population existing in the 'oldest' age group (see Chapter 1). It may be for this reason that early researchers on Antarctic krill concluded that krill only lived for 2-3 years (Bargmann, 1945; Marr 1962). The small (although non-significant) decrease in mean carapace length in this study may represent body shrinkage by individual krill, further complicating the relationship between size and age.

The VB growth curves fitted on carapace length (CL) show that after 6 months of age, the female krill grow at an accelerated rate compared with the male krill. From the model of this laboratory population, female krill grow to a maximum CL of ~13mm by 40 months of age, whereas the male krill reached ~11mm around 32 months of age. For comparative purposes these CLs can be converted to total length of ~39mm (female) and ~35mm (male), [using the equation for female krill:  $AT=11.6 + 2.13CL$  and male krill:  $AT=0.62+3.13CL$  (Goebel, 2007)]. Krill of this size would be classified as 2 years-old, based on a seasonal VB growth curve for wild krill (Candy & Kawaguchi, 2006), or falling into either the 2+ (26-45mm) or 3+ (35-60mm) age groups based on normal distribution analysis (Fach *et al.*, 2006). Either the age of wild krill has been underestimated based on size, or more likely - growth is restricted in the laboratory.

The different sizes attained are likely to be the result of sexual dimorphism in adult krill, but may also represent the male krill switching earlier from somatic growth to reproductive development. Energy may also be diverted, prior to this point, to sex change if protandrous hermaphroditism is occurring. Because sex change in krill has not been reported in the literature, it was assumed in this study that this is not occurring. Interestingly, the divergence in CL between the sexes occurred at ~7mm in this study, compared with ~13mm in wild krill, with the difference between the sexes widening as krill reach maximum length (Goebel *et al.*, 2007). Age and length at maturity has been calculated for wild krill: female krill mature at a total length of ~35mm (estimated to be age 2+ years old), and male krill at ~43mm (3+ years old) (Siegel & Loeb, 1994). The krill in the present study, however, exhibited sexual characteristics at 12 months of age, and the captive male krill did not reach this size at all. It appears that laboratory-kept krill are maturing younger and at smaller sizes than their wild counterparts.

### *Wet Weight of the Eyes*

Previously, eye diameter has been used as a proxy for age in Antarctic krill (Sun & Wang, 1995; Sun *et al.*, 1995; Tarling & Cuzin-Roudy, 2008). This is because the eye is a complex structure, and the hypothesis is that it would be conserved during periods of negative growth (Shin & Nicol, 2002). Eye diameter and the wet weight

of the pair of dissected eyes were measured and compared in the present study. It was found that the wet weight of the eyes provided a more sensitive indicator than eye diameter of increasing eye size – a function of the three-dimensional nature of a sphere versus the two-dimensional nature of a circle. The wet weight of the eyes (WWE) was used, therefore, rather than eye diameter, for further analysis.

There was no difference in WWE attributable to sex, although wild krill with large eyes have been shown previously to be more likely to be male (Tarling & Cuzin-Roudy, 2008). Because there was no sex difference, the data were pooled to fit the VB growth curve, giving a more robust model. The growth curve for WWE did not reach an asymptote in the 48 months of this study, inferring that WWE may continue to increase with age for adult krill, providing a simple tool for age estimation. Further investigation of eye size for krill older than 48 months is needed to confirm this, given that there was no significant difference between WWE at 36 months and 48 months of age.

#### *Age-pigments*

Pigment 1 and pigment 3 (the standardised extracted pigments quantified at wavelengths  $\lambda_{ex}=280\text{nm}$ ,  $\lambda_{em}=625\text{nm}$  and  $\lambda_{ex}=463\text{nm}$ ,  $\lambda_{em}=620\text{nm}$ , respectively) had a positive relationship with age. Pigment 2 (quantified at  $\lambda_{ex}=355\text{nm}$ ,  $\lambda_{em}=510\text{nm}$ ), however, did not have a clear relationship with age, and was not considered to be an age-pigment. This agrees with other published work based on similar methodology for the blue crab *Callinectes sapidus* (Ju *et al.*, 1999), who also found an emission maximum at 510 – 520 nm (excitation at 340-350nm). Ju *et al.* (1999) suggest this pigment is a flavin, present because of the ‘concomitant extraction of flavins known to be present in eye tissue (Fletcher *et al.*, 1973; Davis *et al.*, 1982)’.

Conversely, this pigment has been used as an age-pigment for the krill *Euphausia pacifica*, (Harvey *et al.*, in press). Accumulation of pigment was found to have a positive relationship with age, with increasing variance in the amount of pigment with increasing age (up to 17 months) (Harvey *et al.*, in press). This does, in fact, agree with the data for pigment 2 for *E. superba* in the present study, where there was an increase in the amount of pigment from 12 months to 36 months, with greater variance around the mean at 36 months. The amount of pigment at 48

months however, was significantly lower than at 36 months and with a level not statistically discernable from the amount of pigment at 12 months of age. Over the long time-frame of the present study it is clear that this pigment is not a good predictor of age. It is possible that the early increase in this pigment (apparently with age) is size-dependent.

All three pigments were present at significantly higher amounts in female krill compared to male krill at 12 months of age. The female krill were larger and growing faster than the male krill at this age. Because the pigment values are standardised against protein in the sample, body size alone cannot account for this difference, although it may have been a contributing factor. An alternative explanation is the generation of more pigment as a result of higher metabolism associated with a higher growth rate (the 'rate-of-living' and 'free radical' hypotheses, Chapter 2). Age-pigments accumulate with metabolic time, and hence would accumulate more quickly in a faster-growing organism.

The accumulation of pigment 1 was sex dependent, and separate growth models were developed for the male and female krill. The female krill accumulated pigment 1 more rapidly than the male krill until 26 months of age. After this time, the accumulation of pigment 1 in the female krill slowed, while it continued to increase for the male krill. Pigment accumulation probably reflects biological processes. It is possible to relate initial high accumulation rate in the female krill to high growth rate. The ongoing higher accumulation rate for the male krill may be connected to other biological processes other than growth, such as gametogenesis and increased activity related to reproductive behaviour. Reproductive costs in male krill have been reported to place significant demands on energy reserves, totally depleting triacylglycerol (storage) lipids levels (Virtue *et al.* 1995). The process of mating involves substantial activity in male krill and the increased energetic costs would result in higher pigment accumulation

The quantity of pigment 3 was significantly different for the males and females at age 12 months (as discussed above) and at 48 months at which time male krill had more pigment 3. This may have been a trend consistent with the pigment 1 accumulation model; however, Residual Mean Square (RMS) analysis showed no overall sex effect on the accumulation of pigment 3, so these differences may have been the result of low sample sizes and sampling variation. The VB growth model for pigment 3 was therefore based on pooled data. Pigment

3 also has a positive relationship with age, with accumulation rate slowing with increasing age.

### *Simulation study*

Normally distributed data for each variable (CL - male, CL - female, WWE, and Fig.3) was simulated at ages 12, 24, 36 and 48 months of age, with means and variances obtained from the fit of the VB model for each (Appendix 2). Pigment 1 was not included in this study, as further investigation showed the quantification of this pigment to be inconsistent, compared with pigment 3 (Chapter 6).

Continuation ratio regressions of each variable with age show the poor separation between the curves for each age, particularly for carapace length in the female krill (deviance explained = 8.3%) and male krill (deviance explained = 14.4%). This study shows that carapace length is weakly related to age, for laboratory-kept krill, and that normally distributed size classes could not be used to distinguish between krill aged up to 48 months old.

As expected, there was better separation between simulated age classes based on the wet weight of the eyes (deviance explained = 18.5%), although the relationship is still weak (analogous to  $R^2 = 0.19$ ), and it is difficult to separate age classes based on WWE.

Normally distributed classes based on Pigment 3 had the best separation between ages of all the variables examined (deviance explained = 21.4%). Pigment 3 provides better age discrimination than using wet weight of the eyes, but does not appear to be a practical alternative given the extra cost and work involved in using pigment 3 over the wet weight of the eyes for ageing. Although pigment 3 was the best indicator of age, it is very hard to discriminate between ages 24, 36 and 48 months on this basis. This is due to the high degree of variability about the growth curves, which is the case for all the variables. This could be due to natural variability between krill, plus additional variance due to differences in the coping ability of individual animals to the conditions of captivity. Also, variability in laboratory technique or effectiveness of pigment extraction could contribute to the variance in the pigment data. To illustrate this point, pigment 3 was re-modelled with reduced variance (standard deviation reduced to a third of the estimated

value), which gave good separation between age classes and a deviance explained value of 63.4% (analogous to  $R^2 = 0.63$ ). If it was possible to reduce variance in the data by increasing sample size, reducing natural variability in pigment accumulation (by controlling aquarium conditions more carefully) and eliminate variability in sampling and extraction methodology, pigment 3 would provide a valuable predictor of age for krill.

### *Logistic discriminant analysis*

The discriminant analysis provided a tool to evaluate the usefulness of the variables for predicting age correctly. The analysis is a linear predictor (coded in R, Appendix 3) which is a trade-off of maximising the specificity (getting it right – true positives) against minimising the sensitivity (getting it wrong – false positives) when using variables to predict age. As can be seen from the fit of the VB curves, the differences between all variables at ages 36 and 48 months are not significant enough to be used for discrimination of age groups. At 12 and 36 months old, however, there was enough of a difference to be able to correctly sort the krill a good percentage of the time using carapace length, pigment 1 and pigment 3, particularly when combined with the wet weight of the eyes. Pigment 3 combined with WWE had the best predictive power, correctly sorting 100% of 36 months old krill and 96% of 12 month old krill. Conversely, carapace length correctly predicted the age of all 12 month old krill, but was poorer at correctly sorting the krill that were 36 months olds, only correctly placing 75% of the krill. Pigment 1 was not as useful at correctly determining age at either age class.

For age determination, the best results would be achieved by using a combination of variables: using the wet weight of the eyes significantly increases the power of using age-pigment to predict age. Carapace length, which is the easiest variable to use, can also be useful to age krill. Unfortunately, the relationships that each variable has with age are different, so simply pooling variables does not necessarily lead to better predictive power (e.g see Table 13).

### *Relationship between pigment 3 and wet weight of the eyes*

A criticism that has been made against using extracted pigments for age determination is that apparent accumulation might be a function of increasing organ size, rather than increasing time, *per se* (Sheehy, 2008). To investigate whether this was the case in the present study, quantity of pigment 3 was plotted against the wet weight of the eyes (the organ from which the pigment was extracted). No clear relationship between pigment 3 and WWE could be found, although there may be a weak trend for larger eyes to contain more pigment. The relationship between pigment 3 and age was clear and significant, however, indicating that pigment 3 can be accurately described as an age-pigment.

## **5.6 Conclusions**

Pigments that have a positive relationship with age can be solvent extracted from krill eyes and eyestalks. Pigment 3, which has a spectral peak at  $\lambda_{ex}=463\text{nm}$  and  $\lambda_{em}=620\text{nm}$ , has a stronger relationship with age than carapace length, eye size or the other pigments examined in this study. When combined with the wet weight of dissected eyes, pigment 3 can be used to correctly age mature krill known to be 12 and 36 months 98% of the time. If the variance in the data could be methodologically reduced, the power of using pigment 3 for predicting age increases significantly, and would allow finer discrimination between krill at older age classes.

## Chapter 6: Effects of temperature and diet on growth and the accumulation of age-pigment in Antarctic krill

---

### 6.1 Abstract

The effects of diet and temperature on the accumulation of extractable fluorescent pigments in Antarctic krill, *Euphausia superba*, were experimentally investigated. The effect of laboratory handling was also examined. Wild-caught krill were kept in captivity over a period of 7-12 months, depending on survivorship. The krill were kept at either  $0 \pm 0.3^{\circ}\text{C}$  or  $3 \pm 0.4^{\circ}\text{C}$  and fed one of three diets: food limited, phytoplankton monoculture, or a mixed diet, as described in Chapter 3. Krill from a laboratory population kept under control conditions (Chapter 4) were also sampled for age-pigments over a 12 month period. Pigments were extracted from dissected eyes and eyestalk ganglia, and measured at wavelengths of excitation 280nm, emission 625nm (pigment 1); excitation 355nm, excitation 510nm (pigment 2); and excitation 463nm, emission 620 nm (pigment 3), and standardised against the quantity of protein in each sample. Diet and temperature had significant effects on the change in carapace length (growth) of krill, and in the accumulation of all pigments in krill with time. The increase in quantity of pigment 2 was primarily related to temperature, whereas there were highly significant interactions between diet and temperature with time for the accumulation of pigments 1 and 3. Pigment 3 had the most significant relationship with age, and was the only variable that was significantly affected by handling stress. A comparison of age-pigment results obtained by two independent laboratories was also undertaken using krill from the control population. There was no apparent relationship between pigment 2 and time measured by either laboratory, although there was consistency in the results between the laboratories. There was, however, a positive correlation between pigments 1 and 3 with time over 12 months in the results from both laboratories. A comparison of results between laboratories showed a significant discrepancy in the apparent



accumulation rate of pigment 1. The measured rate of accumulation of pigment 3, however, was consistent between the two independent laboratories. The intercept of the relationship was different between laboratories, possibly due to variation in machine sensitivity. These results further confirm the viability of using extractable age-pigments for age determination in krill. Factors that affect metabolism, such as environmental temperature and diet, which for wild krill are seasonally variable, are important in determining metabolism and hence the accumulation of age-pigment.

## 6.2 Introduction

Fluorescent age-pigments accumulate in animals as a by-product of metabolic processes (Berman, 1989). These pigments accumulate in post-mitotic tissue, such as neurons, over an organism's life span (Katz, 2002; Grune *et al.*, 2005). Measurement of these pigments can be correlated with metabolic time, and perhaps chronological time (O'Donovan & Tully, 1996). Fluorescent age-pigments (FAPs) may therefore prove to be valuable for age prediction in species such as Antarctic krill, which are difficult to age using conventional techniques (Nicol, 2000).

Age pigment accumulation over time in experimental populations has been demonstrated for Antarctic krill (Nicol *et al.*, 1991) and for a variety of other aquatic species: spider crabs (Hirche & Anger, 1988), prawns (Vila *et al.*, 2000), lobsters (O'Donovan & Tully, 1996), crayfish (Sheehy, 1990; 1992; Belchier *et al.*, 1998), fish (Hammer, 1986; Vernet *et al.*, 1988; Hill & Wommersley, 1993) and molluscs (Clarke *et al.* 1990). Despite these demonstrations, the use of FAPs has not been widely adopted for determining age, or for discriminating age classes, partly because of a lack of calibration of these techniques under a wide range of environmental conditions.

Using FAPs for age determination requires calibration of accumulation rate in known-age animals (Chapter 5). Because accumulation of age-pigment is dependent on metabolism, however, factors that affect metabolic rate will influence age-pigment accumulation, and must be investigated to establish the applicability of the model for age prediction in krill developed in Chapter 5.

Metabolism in crustaceans can be influenced by activity (Swadling *et al.*, 2005), food availability (Torres *et al.*, 1994), temperature (O'Donovan & Tully, 1996) and stress (Bergmann *et al.* 2001). Environmental effects, and their associated metabolic repercussions, have been experimentally demonstrated to affect the rate of age-pigment accumulation for a number of species. These include temperature and food ration in fish (Hammer, 1988; Hill & Womersley, 1993), population density and size structure in the crustacean *Homarus americanus* (CORDIS, 2008) and low salinity, high temperature and increasing body mass in a bivalve (Basova *et al.*, 2008). The effects of diet, temperature and stress on growth and pigment accumulation in krill were investigated in the present study.

### 6.3 Materials and methods

#### *Maintenance conditions*

Live krill *Euphausia superba* were collected from Antarctica as described in Chapter 4. The stock krill (control population of ~ 3000 individuals) were kept in a 1000 litre aquarium in the dark at approximately 0°C and fed *Phaeodactylum tricornutum* (ad libitum) for two months, until the krill mortality rate stabilised after the initial capture-stress. A sub-sample of 10 krill were frozen to provide a reference point (time 0) for changes in carapace length and quantity of age pigment. A further sample of 600 krill was then distributed evenly between twelve 20 L containers at 50 krill per container at a stocking density of one krill per 330 ml. Six of the containers were maintained at  $3 \pm 0.4$  °C – referred to as the ‘warm’ treatment, and the other six containers were kept at  $0 \pm 0.3$  °C – referred to as the ‘cold’ treatment. Half of the water in each container was replaced daily with fresh, chilled seawater to improve the oxygen saturation and remove waste. The stocking density was maintained throughout the course of the experiments, so when krill were removed from a container for age-pigment analysis or due to mortality, the water volume was reduced by 330 ml per krill.

Immediately following the daily water change, the krill were fed according to one of three diets; at each temperature the krill in two containers were food-limited, two were fed high levels of the phytoplankton *Phaeodactylum*

*tricornutum*, and two were fed a mixed diet of *P. tricornutum* and a commercial preparation of re-hydrated spray-dried algal cells (Algamac-2000<sup>®</sup>) purchased from Aqua-fauna Bio-marine. The *P. tricornutum* was cultured as described by King *et al.* (2003). The preparation and quantity of food used in these experiments is described in detail in Chapter 4.

#### *Live krill growth measurements*

The krill in the replicate temperature and food treatments were briefly removed from their container once per month (~30 days) and photographed alive for length measurements using digital image analysis (Sigma-ScanPro software by Jandel Corp., 1993-1995). Carapace length (Standard 4, Kirkwood, 1982) and eye diameter was measured. Gender was not observed for the live krill to minimise handling time. To control for handling effects (stress) on growth and pigment accumulation, the krill in the remaining experiments were not handled until they were removed from the containers for pigment analysis.

#### *Age pigment analysis*

Samples of 10 krill were taken for analysis from each container at 1 month, 4 months and 7 months in experimental conditions, unless mortality within the experiment had resulted in reduced krill availability. Krill were sacrificed by placing them within a cryotube which was then frozen in liquid nitrogen. The samples were stored at -86°C. The krill were defrosted, measured and dissected in preparation for pigment extraction as described previously (Chapters 3 & 5). The wet weight of the eyes was recorded. The extraction procedure was modified from Ju *et al.*, (1999) and is described in detail in Chapter 5. The major fluorescence spectra of the extracted pigments were identified and intensities were measured at three pairs of excitation/emission wavelenths:  $\lambda_{ex}=280$  nm and  $\lambda_{em}=625$  nm (pigment 1),  $\lambda_{ex}=355$  nm and  $\lambda_{em}=510$  nm (pigment 2), and  $\lambda_{ex}=463$  nm and  $\lambda_{em}=620$  nm (pigment 3). The fluorescence intensities of the extracted pigments were calibrated against the fluorescence of a standard solution range of quinine sulphate (see Chapter 3).

Protein content was quantified in the samples at  $\lambda_{ex}=280$  nm,  $\lambda_{em}=345$  nm to standardise the amount of pigment measured. The fluorescence intensity of the extracted protein was calibrated using bovine serum albumin (BSA) as a standard (see Chapter 3). The calculated quantity of pigment in each sample was standardized against the quantity of protein, to account for differences in body size and variability in tissue sampling.

Samples from the stock control krill population (see Chapter 4) taken at time of capture (time 0 months), following six months and 12 months in captivity, were analysed for pigment by two independent laboratories (Department of Primary Industries Weribee Laboratories (Victoria, Australia) and University of Maryland Center for Environmental Science (UMCES) (Maryland, USA). The comparative analysis was undertaken to assess the reliability of the technique to produce consistent results. Biochemical analyses were conducted under double-blind procedures; all samples were coded and the laboratories were unaware of the provenance of the samples.

### *Statistical analysis*

Analyses and modeling of the multivariate data were carried out using the software package R and asreml (R Development Core Team, 2008). Summary data, linear models with main effects, linear models with main effects and first order interactions, and Analysis of Variance (ANOVA) were calculated for each of the following parameters: carapace length, wet weight of the dissected eyes, pigment 1, pigment 2 and pigment 3. Tables of coefficients were prepared, along with graphs showing the nature of the interactions of the variables of temperature, diet, stress, sex and time (age) with the response variables pigment 1, pigment 2 and pigment 3, eye diameter, carapace length, wet weight eyes. The models were sequentially reduced based on significant effects.

## 6.4 Results

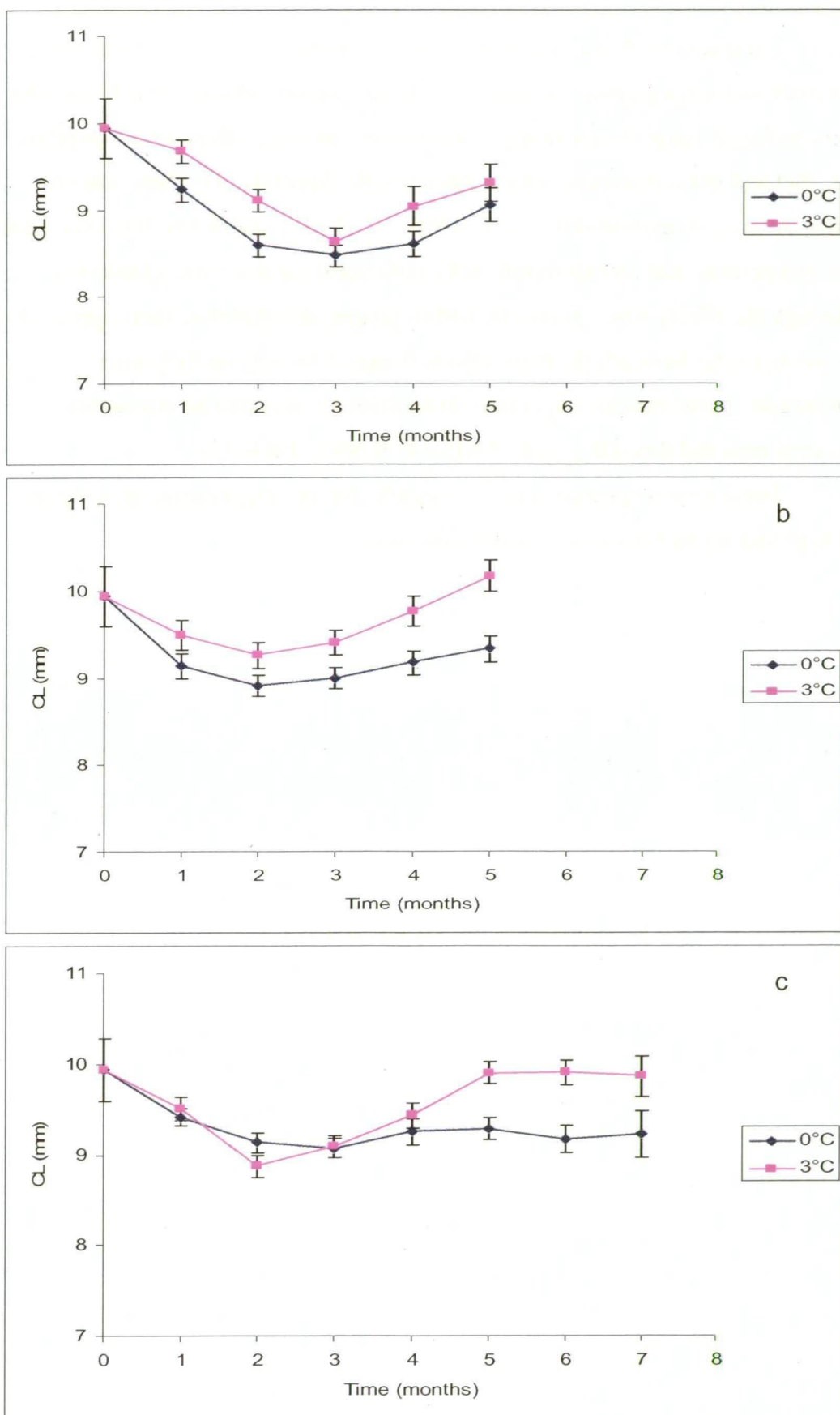
### *Growth*

The growth of the krill in each diet class was plotted from the measurements of carapace length taken on live krill (Figures 30). There was clear separation in size of mean carapace length at 0°C and 3°C for krill that were food limited or fed a phytoplankton diet (Figure 30). Longer carapace lengths were observed in krill maintained at 3°C at all time points (after time 0) under both treatments (food-limited conditions and phytoplankton diets) (Figure 30a and b).

There was no difference in the size of the krill on the mixed diet as a result of temperature in the first 4 months of the experiment, after which time the krill kept at 3°C grew larger than the krill kept at 0°C. Growth ceased by 5 months into the experiment (Figure 30). Krill had the best long-term survivorship on the mixed diet (Chapter 4). It is only in this diet class that krill survived long enough to measure after 5 months (the remainder being used for age-pigment analysis). These graphs (Figure 30a-c) were developed to provide background information about growth within each population. Statistical analysis was carried out for the factors affecting change in carapace length in krill (below), to provide direct comparability with the pigment analysis.

| Component    | DF     | F value | P value | Significant<br>( $\alpha = 0.05$ ) |
|--------------|--------|---------|---------|------------------------------------|
| Time ('age') | 2, 253 | 4.3713  | 0.0136  | **                                 |
| Sex          | 2, 253 | 4.8409  | 0.0086  | **                                 |
| Temperature  | 1, 253 | 5.8989  | 0.0158  | **                                 |
| Diet         | 2, 253 | 3.1979  | 0.0425  | **                                 |
| Stress       | 1, 253 | 0.0987  | 0.7537  | NS                                 |
| Diet.Time    | 4, 229 | 3.6459  | 0.0067  | **                                 |

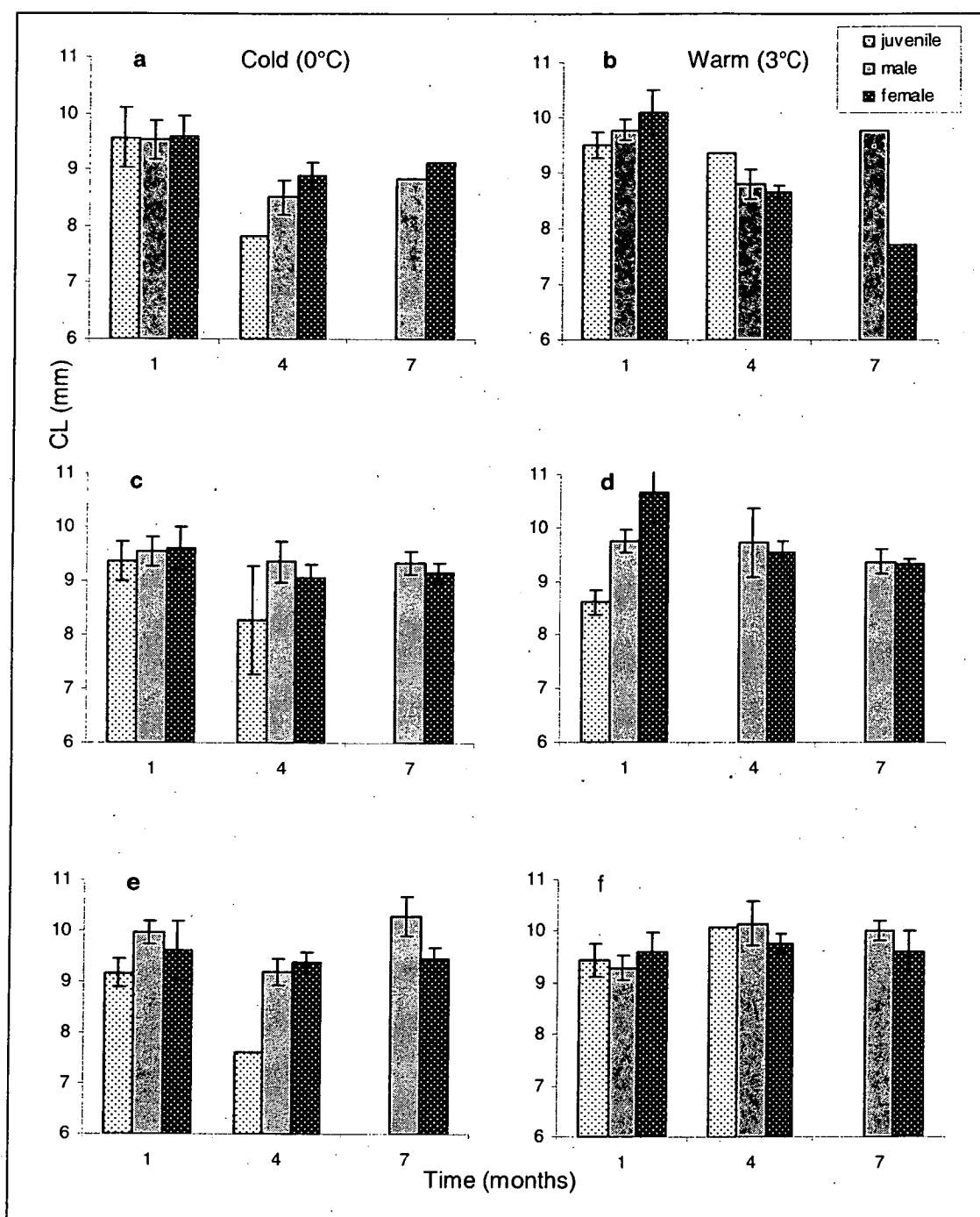
**Table 15.** Summary of coefficients from multivariate analysis (Appendix 4) showing factors that have a significant effect on carapace length of krill. Significant effect is indicated by \*\*. No significant effect is indicated by NS ( $\alpha = 0.05$ ).



**Figure 30.** Mean carapace length (CL)  $\pm$  SE of live krill maintained on (a) a food limited diet, (b) a phytoplankton diet or (c) a mixed diet. Krill were collected from Antarctica in March 2001.

Carapace length was also measured on krill that were sampled from these populations for age pigment analysis at 1, 4 and 7 months (Figure 31). These data were analysed using a linear model to investigate the main effects of temperature, sex, diet and stress (handling) on carapace length (Appendix 4). There was no effect of stress on growth ( $DF_{1, 253}$ ,  $F=0.0987$ ,  $P=0.7537$ , Table 15); however, time, sex, temperature and diet all significantly influenced carapace size (Table 15), although the effects were synergistic within groups, and therefore there appears to be inconsistency between the main effects (Figure 31). Adding first-order interactions to the analysis (Appendix 4) additionally revealed an interaction between time and diet ( $DF_{4, 229}$ ,  $F=3.6459$ ,  $P=0.0067$ , Table 15).

There were no juvenile krill at 7 months for any experiment, showing that all krill had reached sexual maturity by this time.



**Figure 31.** Effects of temperature and diet on carapace length (CL) of juvenile, male and female krill after 1, 4 and 7 months in experiments. Experiments were conducted at 0°C (Figures a, c and e on the left) and 3°C (Figures b, d and f on the right), under food limited conditions (Figures a and b, top row), a mixed diet (Figures c and d, middle row) or a phytoplankton diet (Figures e and f, bottom row).

### *Wet weight of the eyes*

There was no significant effect of sex, temperature, diet, stress or time (1-7 months) on the wet weight of the krill eyes in these experiments (Table 16).



Additionally, there were no significant interactions between measured variables and the weight of eyes (Appendix 4).

| Component    | DF     | F value | P value | Significant<br>( $\alpha = 0.05$ ) |
|--------------|--------|---------|---------|------------------------------------|
| Time ('age') | 2, 253 | 0.6720  | 0.5116  | NS                                 |
| Sex          | 2, 253 | 0.1837  | 0.8323  | NS                                 |
| Temperature  | 1, 253 | 0.6846  | 0.4088  | NS                                 |
| Diet         | 2, 253 | 0.7418  | 0.4773  | NS                                 |
| Stress       | 1, 253 | 1.0839  | 0.2988  | NS                                 |

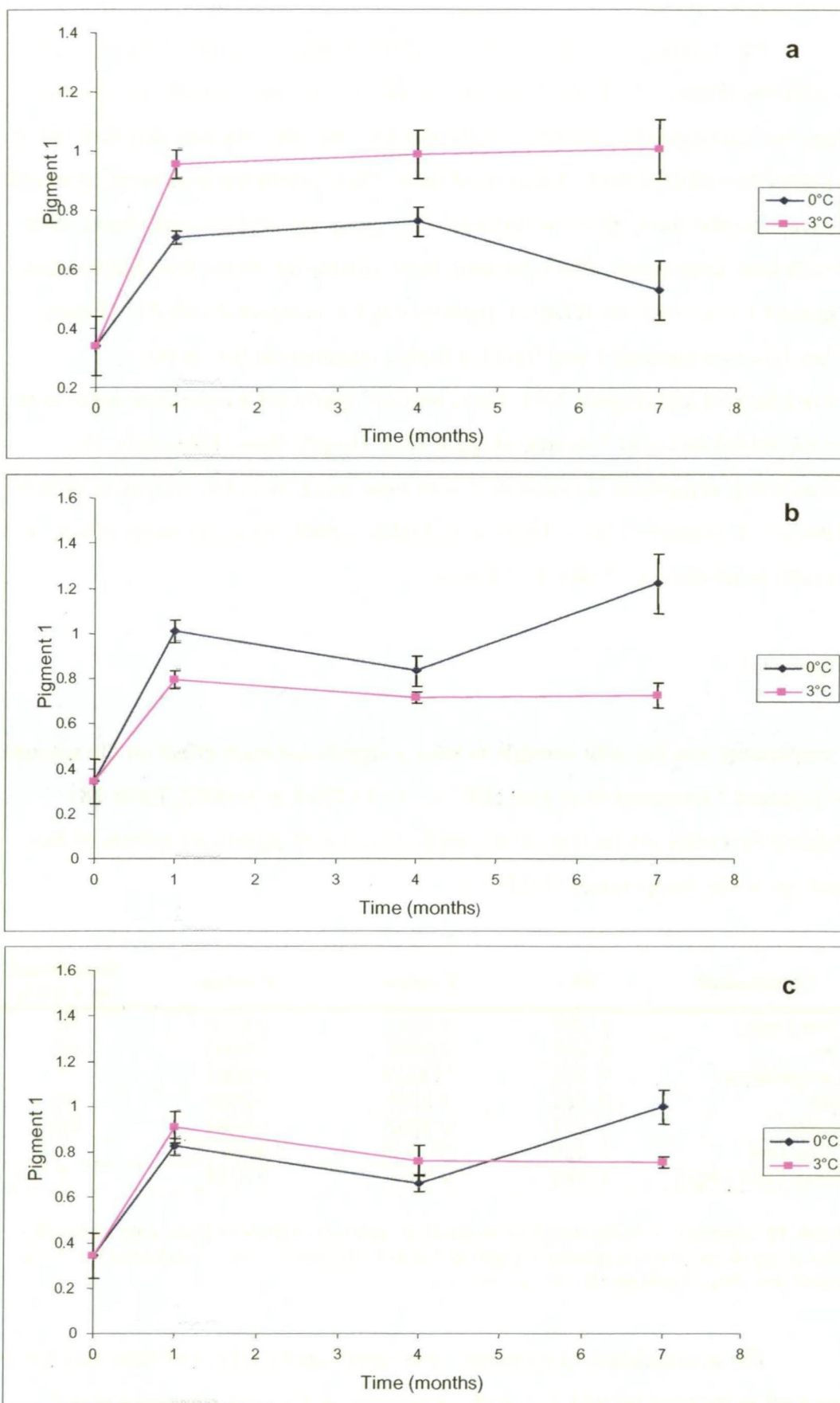
**Table 16.** Summary of coefficients from multivariate analysis (Appendix 4) showing that all components tested had no significant effect on the wet weight of the eyes of krill in this study. No significant effect is indicated by NS ( $\alpha = 0.05$ ).

### *Pigment 1*

Time was the only variable that had a significant effect on the amount of pigment 1 (linear model, Appendix 4), however, adding first order interactions to the analysis revealed significant effects of temperature with time (age), and an interaction between temperature and diet (Table 17). The data are shown plotted by diet to show temperature and time effects (Figure 32a-c).

| Component    | DF     | F value | P value | Significant<br>( $\alpha = 0.05$ ) |
|--------------|--------|---------|---------|------------------------------------|
| Time ('age') | 2, 253 | 7.4608  | 0.0007  | **                                 |
| Sex          | 2, 253 | 1.0463  | 0.3527  | NS                                 |
| Temperature  | 1, 253 | 0.3407  | 0.5599  | NS                                 |
| Diet         | 2, 253 | 3.1979  | 0.2087  | NS                                 |
| Stress       | 1, 253 | 0.0987  | 0.2358  | NS                                 |
| Age.Temp     | 2, 229 | 9.0762  | 0.0002  | **                                 |
| Temp.Diet    | 2, 229 | 23.5995 | 4.8E-10 | **                                 |

**Table 17.** Summary of coefficients from multivariate analysis (Appendix 4) showing variables that have a significant effect on amount of pigment 1 in krill. Significant effect is indicated by \*\*. No significant effect is indicated by NS ( $\alpha = 0.05$ ).



**Figure 32.** Relationship between time and pigment 1 in krill at 0°C and 3°C on a limited diet (a), a phytoplankton diet (b) or a mixed diet (c).

The quantity of pigment 1 increased from time of capture (time 0) in all conditions (Figure 32). In the food limited diet at 0°C, after initially increasing, Pigment 1 significantly decreased with time by 7 months, but was still detected at a higher level than at time of capture (Figure 32a). Similar patterns were observed between temperature effects within each diet group up until 4 months time, after which time temperature effects became more significant. In the food limited diet, pigment 1 was observed at higher quantities at 3°C compared with 0°C (Figure 32a), however pigment 1 was found at higher quantities at 0°C in the phytoplankton diet (Figure 32b). There was no significant temperature separation on the mixed diet until 7 months (Figure 32c). Despite these differences, the relationship of pigment accumulation with time was reasonably similar in all three diets at 3°C (Figures 32a-c). There were highly significant temperature effects at 7 months in all diets ( $t=-3.648$ ,  $P=0.0003$ ).

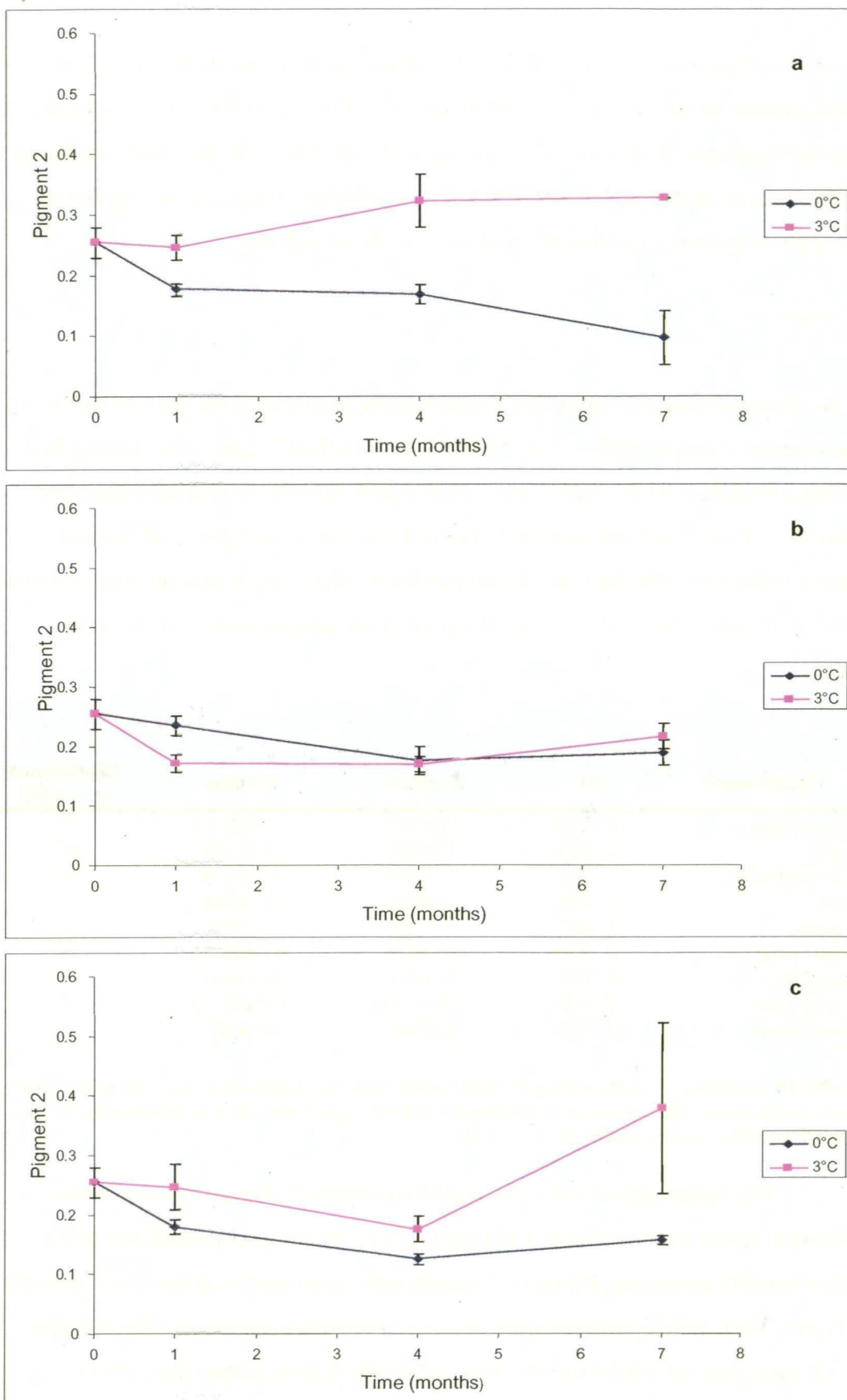
## Pigment 2

Temperature was the only variable to have a significant main effect on the amount of pigment 2 measured from krill ( $DF_{1,253}$ ,  $F=13.8224$ ,  $p=0.0002$ , Table 18). Adding first order interactions to the analysis revealed significant effects of diet and age within temperature (Table 18).

| Component         | DF     | F value | P value   | Significant ( $\alpha = 0.05$ ) |
|-------------------|--------|---------|-----------|---------------------------------|
| Time ('age')      | 2, 253 | 2.2027  | 0.1126    | NS                              |
| Sex               | 2, 253 | 0.0950  | 0.9093    | NS                              |
| Temperature       | 1, 253 | 13.8224 | 0.0002    | **                              |
| Diet              | 2, 253 | 1.3280  | 0.2669    | NS                              |
| Stress            | 1, 253 | 0.3262  | 0.5684    | NS                              |
| Temp.Diet         | 2, 229 | 10.2612 | 5.397E-05 | **                              |
| Temp.Time ('Age') | 2, 229 | 4.6372  | 0.0106    | **                              |

**Table 18.** Summary of coefficients from multivariate analysis (Appendix 4) showing factors that have a significant effect on amount of pigment 2 in krill. Significant effect is indicated by \*\*. No significant effect is indicated by NS ( $\alpha = 0.05$ ).

The accumulation of pigment 2 was significantly related to time only for the krill in the food limited diet, with an increase at 3°C and a decrease at 0°C ( $DF_{2,229}$   $F=4.6372$ ,  $P= 0.0106$ , Figure 33a). There was no significant change in the



**Figure 33.** Relationship between time and pigment 2 in krill at 0°C and 3°C given a limited diet (a), a phytoplankton diet (b) or a mixed diet (c).

quantity of pigment 2 for krill in the phytoplankton diet, and there was no effect of temperature for this group. For krill on the mixed diet, pigment 2 was found in higher quantities at all times at 3°C compared with 0°C, with the most significant difference occurring at 7 months ( $t=3.325$ ,  $P=0.0010$ ). There was no significant change in quantity of pigment 2 with time in the mixed diet.

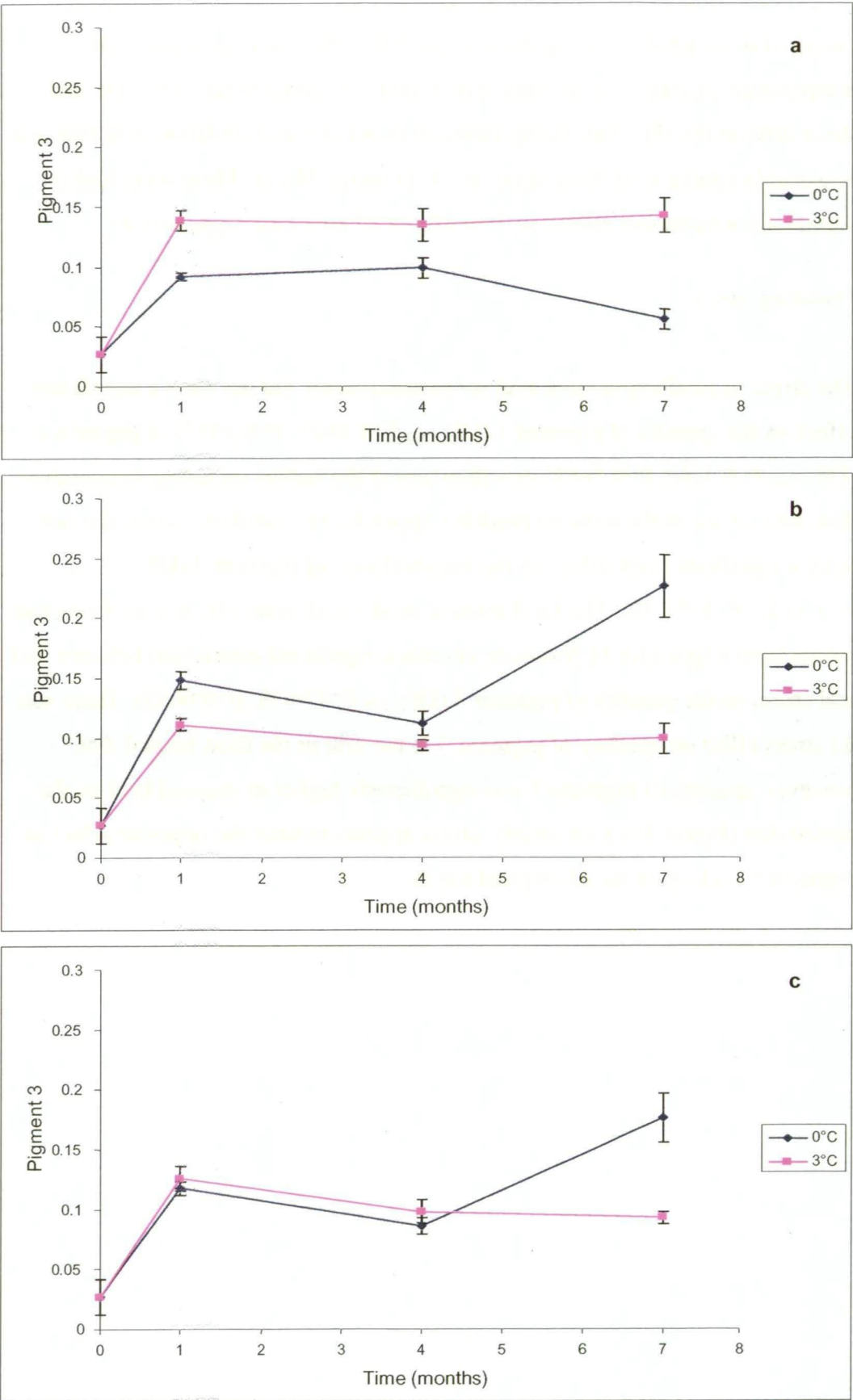
### *Pigment 3*

The observed amount of pigment 3 significantly increased from time of capture (0 months) to 7 months ( $DF_{2,253}$ ,  $F=17.0167$ ,  $P=1.165E-07$ , Table 19). Adding first order interactions to the analysis revealed significant effects of temperature and diet at each time, and interactions between temperature and diet, and diet and stress (Table 19). The data are shown plotted by diet to show temperature and time effects (Figure 34a-c). The stress effects are dealt with separately in the next section.

| Component    | DF     | F value | P value   | Significant<br>( $\alpha = 0.05$ ) |
|--------------|--------|---------|-----------|------------------------------------|
| Time ('age') | 2, 253 | 17.0167 | 1.165E-07 | **                                 |
| Sex          | 2, 253 | 0.4926  | 0.61165   | NS                                 |
| Temperature  | 1, 253 | 3.2738  | 0.07158   | NS                                 |
| Diet         | 2, 253 | 1.9358  | 0.14644   | NS                                 |
| Stress       | 1, 253 | 1.1182  | 0.2913    | NS                                 |
| Age.Temp.    | 2, 229 | 22.7425 | 9.790E-10 | **                                 |
| Age.Diet     | 4, 229 | 2.9971  | 0.01943   | **                                 |
| Temp.Diet    | 2, 229 | 24.8220 | 1.750E-10 | **                                 |
| Diet.Stress  | 2, 229 | 4.9058  | 0.0082    | **                                 |

**Table 19.** Summary of coefficients from multivariate analysis (Appendix 4) showing factors that have a significant effect on amount of pigment 3 in krill. Significant effect is indicated by \*\*. No significant effect is indicated by NS ( $\alpha = 0.05$ ).

The accumulation rates observed for pigment 3 (Figures 34a-c) follow identical trends as for pigment 1 (Figures 32a-c). In the food limited diet at 0°C, after initially increasing, Pigment 3 significantly decreased with time by 7 months (Figure 34a). Similar patterns were observed between temperature effects within each diet group up until 4 months time, after which time temperature effects became more significant. In the food limited diet, pigment 3 was observed at higher quantities at 3°C compared with 0°C (Figure 34a), however the opposite

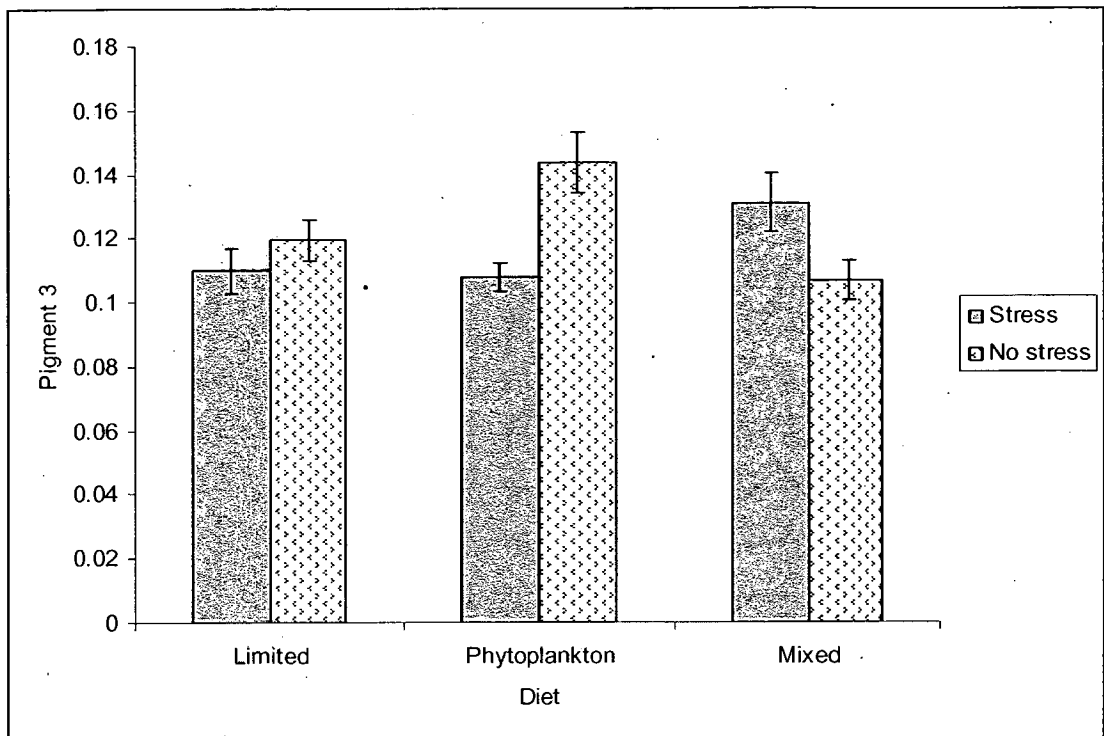


**Figure 34.** Relationship between time and pigment 3 in krill at 0°C and 3°C given a limited diet (a), a phytoplankton diet (b) or a mixed diet (c).

occurred in the phytoplankton diet (Figure 34b). There was no significant temperature separation on the mixed diet until 7 months (Figure 34c). Despite these differences, the relationship between pigment 3 accumulation with time was reasonably similar in all three diets at 3°C (Figures 34a-c). There were highly significant temperature effects at 7 months in all diets (see Appendix 4).

### *Handling stress*

The stress of handling the live krill for measurements did not have a significant effect on the quantity of pigment 1 ( $DF_{1, 253}$   $F=1.4125$ ,  $P=0.2357$ ) or pigment 2 ( $DF_{1, 248}$   $F=0.3262$ ,  $P=0.5684$ ) or within any of the factors including temperature, diet, time or sex in the reduced models (Appendix 4). Similarly, stress did not have a significant main effect on the accumulation of pigment 3 ( $DF_{1, 253}$   $F=1.1182$ ,  $P=0.29130$ ). The fit of a linear model with main effects plus first order interactions (Appendix 4), however, reveals a significant interaction between diet and stress on the quantity of pigment 3 ( $DF_{2, 229}$   $F=4.9058$ ,  $P=0.0082$ ). There was no stress effect on quantity of pigment 3 in the krill in the food limited diet; however, quantity of pigment 3 was significantly higher in stressed krill on the mixed diet (Figure 35). Conversely, stress appears to have the opposite effect on pigment 3 for krill on the phytoplankton diet.



**Figure 35.** Effects of handling stress on mean quantity of pigment 3 with different diets at all times.

#### *Assessment of the reliability of the extraction technique*

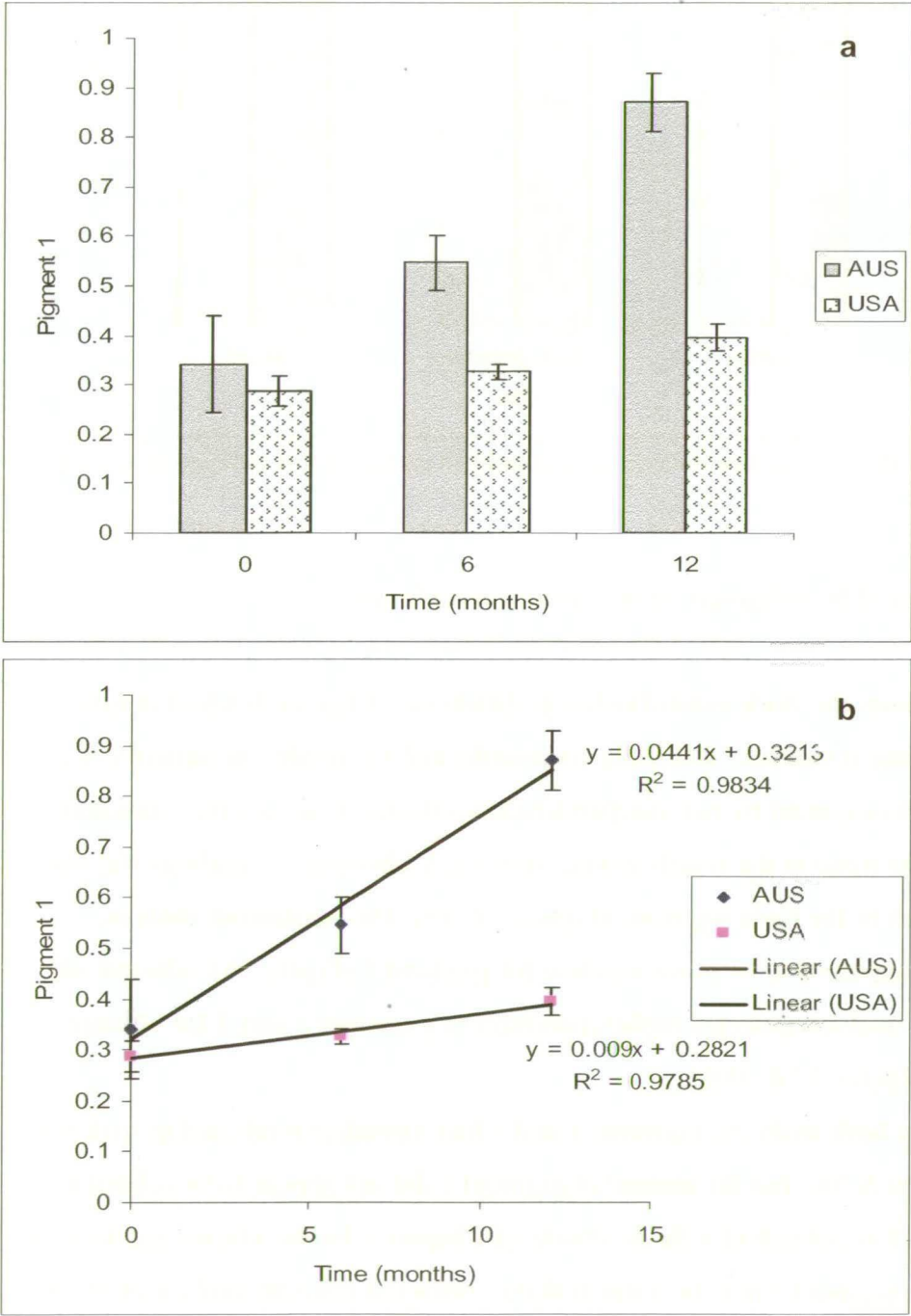
Samples from the stock control krill population (see Chapter 4) taken at time of capture (time 0 months), following six months and 12 months in captivity, were analysed for pigment by two independent laboratories, to assess the consistency of results. The trend in the results produced by each laboratory's analysis was similar within each of the three pigments (Figures 36-38). The Australian analysis, however, appeared to be more sensitive for pigment 1 (Figure 36), whereas the American analysis detected higher quantities of pigments 2 and 3 for all time classes (Figures 37 & 38).

For both analyses, pigments 1 and 3 had a positive relationship with time (Figures 36 & 38), but the amount of pigment 2 did not appear to be related to time, which is consistent with the results of Chapter 5 for the known age krill. As discussed, pigment 2 may be a flavin that is extracted concomitantly with the true age-pigments, and would not be expected to correlate with time (Chapter 5).

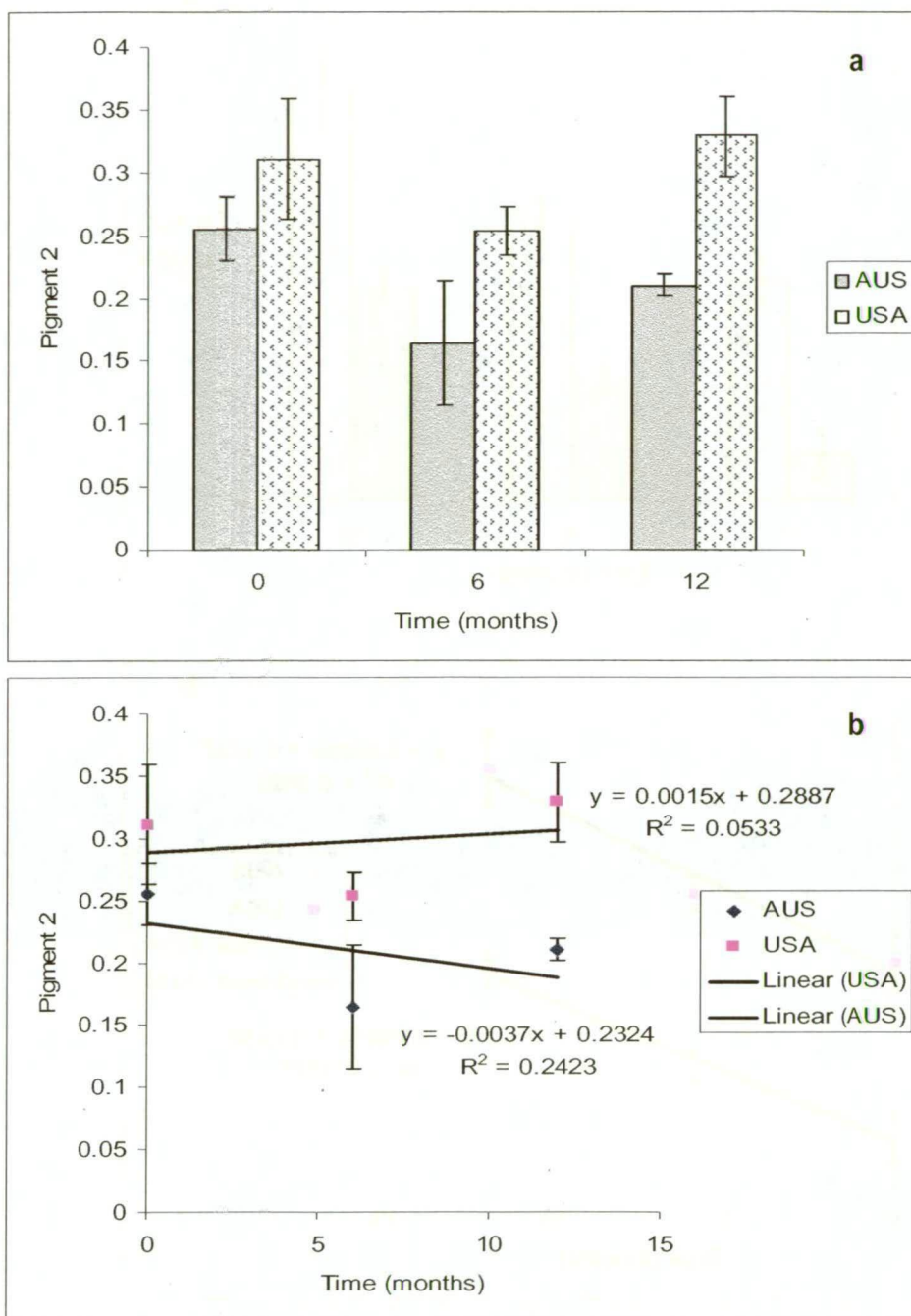
Linear regressions were fitted to all relationships for the purpose of comparing the ability of the extraction technique to produce consistent results (equations and  $R^2$  values on Figures 36b, 37b & 38b). Consistency in detection of



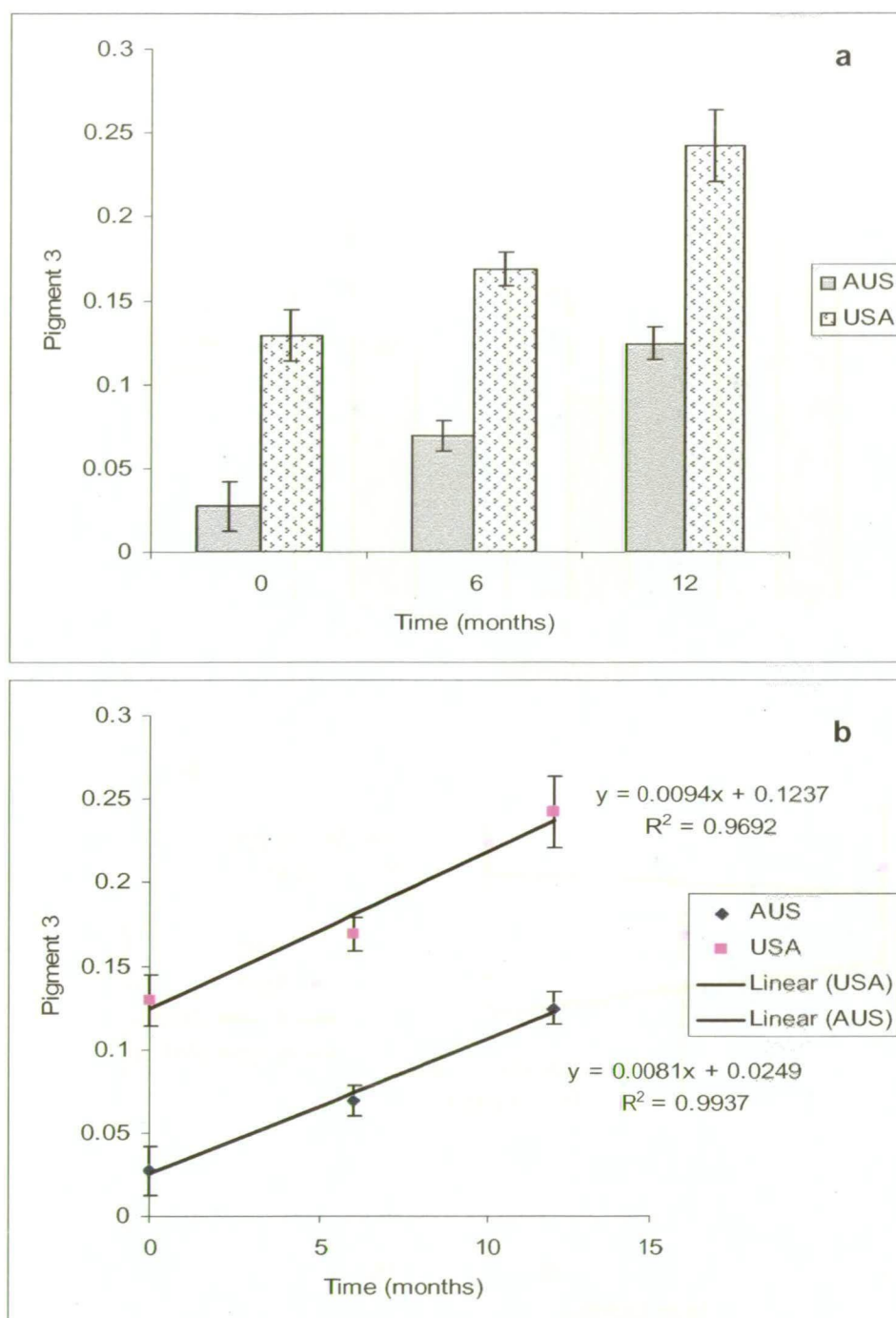
the rate of accumulation of pigment was only achieved between the two laboratories for pigment 3 (Figure 38b). The accumulation of pigment 3 can be described by the equation  $y = 0.0094x + 0.1237$  ( $R^2 = 0.9692$ ) for the American analysis, or by the equation  $y = 0.0081x + 0.0249$  ( $R^2 = 0.9937$ ) for the Australian analysis.



**Figure 36.** A comparison of quantity of pigment 1 measured at Ex 280/Em 625nm in analogous sub-samples over 12 months (a), and the linear regressions of pigment accumulation with time (b). Extracted krill pigments were analysed by two independent laboratories (Department of Primary Industries Weribee Laboratories (Victoria, Australia) and University of Maryland Center for Environmental Science (UMCES) (Maryland, USA)).



**Figure 37.** A comparison of quantity of pigment 2 measured at (Ex 355/Em 510 nm) in analogous sub-samples over 12 months (a), and the linear regressions of pigment accumulation with time (b). Extracted krill pigments were analysed by two independent laboratories (Department of Primary Industries Weribee Laboratories (Victoria, Australia) and University of Maryland Center for Environmental Science (UMCES) (Maryland, USA)).



**Figure 38.** A comparison of quantity of pigment 3 measured at Ex 463/Em 620nm in analogous sub-samples over 12 months (a), and the linear regressions of pigment accumulation with time (b). Extracted krill pigments were analysed by two independent laboratories (Department of Primary Industries Weribee Laboratories (Victoria, Australia) and University of Maryland Center for Environmental Science (UMCES) (Maryland, USA).

## 6.5 Discussion

### *Growth*

It is well established that krill growth is strongly dependent on temperature and nutrition (see Buchholz, 2003). Growth trajectories based on measurements of live krill showed clear differences related to temperature and diet in the present study. Longer carapace lengths were recorded at the warmer temperature of 3°C for krill on a food limited or phytoplankton diet, and after five months for krill on a mixed diet. Diet was more important than temperature, however, for determining carapace length from 1-4 months for krill fed a mixed diet. Additionally, it has been demonstrated previously that quantity of food can obscure more subtle effects of food quality for growth of wild krill (Atkinson *et al.*, 2006).

Initial growth was negative for krill in all conditions in this study. Since the krill were collected at the beginning of autumn this may reflect a seasonal effect, because it occurs in all treatments. Positive growth was observed from 3 months onwards for krill on a food limited diet and those fed a mixed diet at 0°C, and from 2 months onwards for the krill fed phytoplankton and the mixed diet at 3°C. Live krill were repeatedly measured to generate these growth curves, so it is likely that body shrinkage in the live krill, rather than mortality of larger krill, was the most important factor in determining the reduction in observed carapace length.

At 3°C (all diets) the female krill were initially larger than the male krill (at time 1 month), however male krill were larger than the female krill at 4 and 7 months, possibly reflecting differential growth rates, consistent with published results (Tarling *et al.*, 2006, Virtue *et al.* in press).

As expected, statistical analysis of the carapace length measurements taken from krill used for age pigment analysis in the present study, showed significant effects of temperature, sex and diet. Handling stress did not have a measurable effect on change in carapace length.

The purpose of these analyses was to provide information about the effects of temperature and diet on the growth of krill in these experiments, because they are integrally linked with metabolic processes and therefore age-pigment accumulation.

Krill have compound eyes, which are complex organs (Odselius & Eloffson, 1981). The crustacean compound eye has ommatidia (eye facets) with focusing crystalline cones, and colour and/or polarisation vision (Nilsson & Kelber, 2007), and grow at the margins by the addition of new ommatidia (Mayer, 2006). It has been demonstrated that the krill eye (number of ommatidia, optical neurons, and therefore size) is conserved during periods of zero or negative growth, probably due to its complex structure (Sun *et al.*, 1995; Shin & Nicol, 2002).

It has been demonstrated that the wet weight of the dissected eyes (WWE) has a more sensitive relationship with age than eye diameter, and that WWE continues to increase with age in adult krill (Chapter 5). It was necessary, therefore, to investigate the effects of sex, temperature, diet and stress on WWE, if eye weight is to be used as a proxy for age. There were no significant effects, or interactions, of any of these variables on the wet weight of the krill eyes in the present study, which gives WWE increased robustness of applicability. There was no detectable change in WWE over 7 months, either, so it might be possible that environmental variables may have a significant effect on eye size over a longer period of time than was investigated in this study. That there was no change in WWE during a period of negative growth supports the theory of eye conservation described above. It has been demonstrated that eye diameter of starved krill does not decrease, even when animals have shrunk in body length, and that eye diameter of well-fed krill continued to increase as overall length increases, over 7 months of investigations (Shin & Nicol, 2002). It was found that this created a distinction between fed and starved krill, that could not be distinguished based on body length alone. Additionally, eye growth of krill recommenced with re-growth of krill following shrinkage following a time lag (Shin & Nicol, 2002). No significant change in WWE was found in the present study over the same period of time; this is probably related to body shrinkage followed by re-growth, over which time no change in eye size would be expected.

### *Sex effects on pigment accumulation*

There were no detectable effects of sex on the accumulation of any of the three pigments. This is an interesting result, because male and female krill have significantly different growth patterns and rates (Tarling *et al.*, 2006; Kawaguchi *et al.*, 2006; Virtue *et al.*, in press, Chapter 5 and this study). Because growth and metabolism are inextricably linked (e.g. Iguchi & Ikeda, 1995), and it is thought that pigments 1 and 3 are age-pigments and hence metabolically derived, a sex-related effect was expected. The amount of pigments 1 and 3 were found to be sex dependent over a longer period time for known-age krill (Chapter 5). It is likely, therefore, that the present study did not detect this effect due to natural variance in the initial amount of pigment for krill of either sex. This is because wild-caught krill of indeterminate age were used, which possibly included some variability in ages within each sex class.

### *Temperature and diet effects on pigment accumulation*

The effects of temperature and diet on the accumulation rate of pigments in krill had a complex, interdependent relationship, and it is difficult to separate the effects of diet from temperature. Differences in temperature had a significant overall effect on the accumulation of pigment 2, but not pigment 1 or pigment 3. Analysis of first order interactions, however, revealed that the temperature differences in the accumulation rates of pigments 1 and 3 were present within time and diet classes. That temperature is the primary determinant of pigment 2 quantity, but not the other pigments, suggests that they might have different mechanisms of formation. This supports the idea that pigment 2 belongs to a different class of pigments. It has been proposed (Ju *et al.* (1999) & Chapter 5) that this pigment ( $\lambda_{\text{ex}} \sim 340\text{-}350$ ,  $\lambda_{\text{em}} \sim 510\text{-}520$ ) may belong to a class of pigments such as flavins, which are yellow pigments based on pteridine (Voet & Voet, 2004) and known to be in eye tissue (Fletcher *et al.*, 1973; Davis *et al.*, 1982). Interestingly, the fluorescence of pteridine has been used with some success to age insects (Robson *et al.*, 2006).

The effect of temperature on the accumulation of pigment 1 and pigment 3 had almost identical trends: when food was limited there were significantly higher

levels of pigments 1 and 3 at the warmer treatment of 3°C compared to 0°C, at all times post-capture. A positive relationship of age-pigment with temperature was expected, given the known effects of temperature on growth and metabolism (see section on growth above). Temperature has been shown to increase age-pigment accumulation in the lobster *Homarus gammarus* (O'Donovan & Tully, 1996), the pike, *Esox lucius* (Hammer, 1988) the milkweed bug *Oncopeltus fasciatus* (McArthur & Sohal, 1982) and the stomatopod *Oratosquilla oratoria* (Kodama *et al.*, 2006).

Conversely, more age pigment was recorded for krill in the cold treatment of 0°C when fed the phytoplankton diet (all times), and the mixed diet (at 7 months). Temperature has also been shown to have a complex relationship with the accumulation of age-pigment with a thermal optimum for the crayfish *Cherax quadricarinatus* (Sheehy *et al.*, 1995). It appears from the results of the present study, that processes leading to age-pigment accumulation are very sensitive to temperature, and that metabolic compensation may have an influence on the observed relationships between pigment and temperature. While increasing temperature correlates with increasing metabolism and growth, wild krill have been demonstrated to have the highest growth rates at 0.5°C (Atkinson *et al.*, 2006) representing a trade-off between optimum temperature for metabolic processes and thermal stress. Individual Antarctic krill, however, may never experience great fluctuations in temperature during their life times if they remain in a single geographic location, because seasonal cycles in water temperature and vertical gradients are less extreme than in other areas. For example, *Meganyctiphanes norvegica* may experience daily changes in water temperature of over 10°C as a result of vertical migration (Tarling *et al.*, 1999; Saborowski *et al.*, 2000) yet for much of the Southern Ocean, the difference in temperature between the surface and deeper waters is rarely more than 2°C (e.g. Bindoff *et al.*, 2000). This relatively stable thermal environment might make Antarctic krill a good subject for further studies on age-pigment accumulation in wild populations.

Because the quantity of pigment 3 was measured at an order of magnitude lower than the quantity of pigment 1, it is difficult to directly compare the amount of separation of pigment accumulation based on temperature difference, however it appears that pigment 3 is more sensitive than pigment 1 to temperature at 7 months (greatest divergence in means at the longest time under experimental conditions).

Differences in temperature may therefore account for quite significant variance in the quantity of age-pigment for krill at a given age.

There were clear differences in pigment accumulation in krill caused by diet. In the food limited diet, temperature had more significant effects on pigment accumulation, than in either of the phytoplankton or mixed diets: pigment accumulation rates diverged for all three pigments from 1 month into the experiments, compared with 4 months for the krill fed phytoplankton or a mixed diet (except for pigment 2 on the phytoplankton diet, where there was no overall temperature effect). This may be because the krill were physiologically stressed by lack of food quantity and quality, and were therefore less thermally adaptive, which would typically manifest as higher respiration rates at higher temperatures, leading to higher metabolic rate and a higher rate of age-pigment accumulation.

Temperature adaptation is an essential physiological phenomenon for poikilothermal organisms such as krill, which are dependent on environmental temperatures (Lahdes, 1995). Physiological adaptation to thermal changes in the northern krill, *Meganyctiphanes norvegica*, has been investigated using oxygen consumption rates, showing that krill did not physiologically adapt to temperature changes. It was postulated that the krill used behavioural adaptations to cope with temperature gradients in the environment (Saborowski *et al.*, 2000).

The relationships of pigment accumulation with the phytoplankton diet and the mixed diet were similar, but with temperature having a more pronounced effect in the phytoplankton diet. It was expected that there would be more significant differences in pigment accumulation observed between krill fed the phytoplankton monoculture diet and the mixed diet. That temperature had a more significant effect in the phytoplankton diet suggests that there is a component missing from this diet, (compared to the mixed diet) which would otherwise enable the krill to cope better with thermal stress. The added component of the mixed diet that would be responsible for nutritional differences between these diets is the commercial preparation of *Schizochytrium sp* (Algamac-2000®). This product contains high levels of protein, essential fatty acids, vitamins and carbohydrates (Appendix 5). Increasing nutritional value by adding this preparation to the diet may have reduced the overall variance in age pigment accumulation over time. Because a mixed diet (rather than a monoculture diet) is more similar to the natural diet of wild krill age, pigment accumulation in wild krill may be less variable than that of



laboratory kept krill, strengthening the use of extractable pigment for age determination of wild krill.

There is evidence that diet plays an important role in age-pigment formation. Dietary restriction has been shown to slow accumulation rate (Katz et al., 1993), and the presence of antioxidants, such as vitamins A, C and E, in the diet have shown mixed effects. Vitamin E in the diet reduces age-pigment accumulation, which is attributed to the reduction of reactive oxygen species (ROS) in the body, hence slowing cellular ageing (Kruk & Enesco, 1981; Fattoretti, 2002). A combination of vitamins C and E added to diet have shown similar effects for the shrimp, *Penaeus japonicus* (Castro et al., 2002). Conversely, experimental supplementation of diet with vitamin A has led to increased age-pigment generation (Radu et al., 2008). This suggests that age estimation of wild krill, based on pigment accumulation in a laboratory population, may be biased where wild krill inhabit regions of diverse feeding and temperature conditions. Clearly, a relationship between diet and age-pigment exists and is complex, hence warranting further investigation to understand the metabolic processes involved.

#### *Stress (handling) effects on pigment accumulation*

Stress was experienced by the krill due to handling once per month for morphometric measurements. This was only detectable in the accumulation of pigment 3 for krill fed the phytoplankton and mixed diets. The food-limited krill were probably under high levels of physiological stress which masked the additional stress of being handled. The quantity of pigment 3 was significantly higher in stressed krill compared with unstressed krill that received the mixed diet. It would be expected that stress would result in an increase in pigment 3, as handling stress (increased exercise and aerial exposure) can increase aerobic metabolism (e.g. Bergmann et al., 2001) which leads to age-pigment formation. Conversely, in the present study stress appears to have the opposite effect on pigment 3 for krill on the phytoplankton diet. This may possibly be the result of longer term effects of stress (slower recovery, suppression of activity, lower metabolism) in the days following handling. There have been no experiments that have specifically addressed the issue of handling stress on krill, but there are

reports of increases in metabolism associated with activity (eg. Swadling *et al.*, 2005; Johnson & Tarling, 2008). There is also considerable literature on the various effects of environmental variables on the metabolism of marine animals (eg. Seibel & Drazen, 2007).

#### *Assessment of the reliability of the extraction technique*

Pigments were measured at three sets of wavelengths that represented the peak fluorescence intensities detected in the extract by a scanning fluorescence detector. The relationships of these pigments with age have previously been investigated to identify which might be age-pigments (Chapter 5). In the present study, pigments 1 and 3 were found by both laboratories to have a relationship with time, but pigment 2 did not. This pigment has been reported previously to be a flavin (Ju *et al.*, 1999).

Consistency in detection of the rate of accumulation of pigment was only achieved between the two laboratories for pigment 3. The value of the intercept is clearly different for the two analyses, but this is very likely a result of machine sensitivity, which can be taken into account to make the data comparable. Importantly, the detection of accumulation rates of pigment 3 is the same between the laboratories. This is an important result, since pigment 3 was the most valuable predictor variable of age.

## **6.6 Conclusions**

Environmental parameters in the laboratory culture facilities used in this study have been demonstrated to influence growth and age-pigment formation of krill. Temperature and diet have significant and measurable effects on the accumulation of pigments with time, and the relationship is complex and synergistic. Increasing nutrition, in terms of food quantity and quality, appears to increase the ability of krill to cope with thermal variability. These factors partially contribute to the variance in age-pigment quantity in krill of the same age. The age pigment extraction technique developed for this research produced consistent and therefore directly comparable results for pigment 3 when used in different laboratories. The

results here prove this method to be a valuable tool, in conjunction with other demographic information, to measure krill age.

## Chapter 7: General Discussion

---

The Antarctic krill, *Euphausia superba*, is an ecologically and commercially important species. Basic demographic information about krill is required for the model that is used to regulate the fishery and protect the ecosystem; however, there remain uncertainties surrounding the age structure and longevity of wild populations. Bias in the methods currently used to estimate age and growth rates results in high levels of uncertainty in stock assessment.

In the current study, investigations using extractable fluorescent pigments in Antarctic krill of known-age produced the first calibration of pigment vs. age for this species. Environmental factors that affect metabolism were found to influence age-pigment accumulation in the laboratory; however, despite the subsequent high variance in observed quantities of pigment in each age class, age pigments produced an improved method for age determination when compared to simple measures of size (carapace length).

Age classes within a population have generally been surmised by analysing length frequency data for cohorts (Siegel & Nicol, 2000). There are many problems associated with this method, including: sampling bias which might lead to underestimation of sizes (Marr, 1962) or over-estimation of sizes (Hill *et al.* 1996; Reid, 2001), measurement bias (Watkins *et al.*, 1985), movement of krill into and out of local populations (Reid *et al.*, 1999), arbitrary decisions about separation of cohorts for data analysis (Nicol, 2000), body shrinkage (Ikeda & Dixon 1982, Sun *et al.*, 1985) and size-dependant mortality (Alonzo *et al.*, 2003). Laboratory studies have not improved knowledge on the relationship between length and age because of uncertainties in extrapolating these studies to wild populations (Nicol, 2000), and in fact, have served to highlight unsuspected problems such as shrinkage (Ikeda and Dixon, 1982). Because of these multiple problems, alternative approaches to estimating the age of wild-caught krill have been tried including multiple morphometrics (Farber Lorda, 1991), eyeball diameter (Sun *et al.*, 1995) and the use of fluorescent pigments that accumulate with age (Ettershank, 1984). Ettershank clearly laid out the problems of the

conventional age estimation techniques for krill, and initiated a series of studies on ageing of krill that has resulted in the current thesis. Although there were problems with the original methodology described by Ettershank (e.g. Nicol, 1987), subsequent methodological developments have resulted in the techniques that have proved effective in other species, and which were used in this thesis. Fluorescent age-pigments can therefore provide a complimentary tool for age-determination in species such as krill that are difficult to age from length data with any certainty.

Pigment accumulation with age has been demonstrated for a range of crustaceans, including lobsters (O'Donovan & Tully, 1996; Sheehy *et al.*, 1998) spider crabs (Anger, 1983; Hirche & Anger, 1988), freshwater crayfish (Sheehy, 1990c; 1992; Belchier *et al.*, 1998), prawns (Sheehy *et al.*, 1985; Vila *et al.*, 2000), blue crabs (Ju *et al.*, 1999, 2001, 2003), Antarctic shrimp (Bluhm & Brey, 2001) and Antarctic amphipods (Bluhm *et al.*, 2001). Age-pigments have also been demonstrated to accumulate in krill (Nicol *et al.*, 1991), however this research has been hampered by technical difficulties with the extraction method (see Nicol, 1987), lack of measurable results for the histological method (Bluhm *et al.*, 2001), and more importantly the lack of calibration with known age animals over a range of environmental variables. Additionally, long-term rearing studies for ageing purposes have only recently become possible for krill (Hirano & Matsuda, 2003) because of the difficulty of keeping groups of krill alive in captivity over long periods of time (Ikeda & Thomas, 1987). Individual krill have been maintained over long periods in captivity (Ikeda & Thomas, 1987), but population age studies require large numbers of captive krill.

With the development of a refined biochemical extraction technique using dichloromethane-methanol as the solvent, pigments have been analysed using an HPLC auto-sampler to successfully age blue crabs (Ju *et al.*, 1999, 2001, 2003). This automated analysis, used with a scanning fluorescent detector to identify fluorescent maxima, has facilitated the analysis of a large number of samples. Combined with the development of long-term rearing of krill populations in captivity, the improvement of the extraction technique provided the opportunity to further develop this ageing technique for krill in this thesis.

Two of the three pigments extracted from krill eyes and eyestalks in the present study (using the method developed by Ju *et al.*, (1999, 2001, 2003)) were found to accumulate with age over 48 months in individuals from a population that

had been reared from eggs hatched in an aquarium at a known date. This data has allowed calibration of the pigment accumulation rate with age, and the development of a model that can be used to predict age from the measured quantity of pigment. The usefulness of using the quantity of pigment to predict age was compared to that of using two other methods of ageing krill: carapace length and the wet weight of the eyes. Age-pigments had more power in correctly predicting age than either carapace length or the wet weight of the eyes, however the extraction and analysis of these fluorescent pigments make this method more difficult and expensive to routinely use. Additionally, high variance in pigment quantity within each age class was found. Significant and unexplained variation in pigment content between animals within an age class maintained under similar conditions has been observed before (Sheehy, 1990a). In the present study this variation was attributed to natural variability in individuals due to genetic differences and metabolic adaptability (Hill & Womersley, 1993), variance due to small fluctuations in environmental conditions, (temperature, diet and stress), and variance due to small-scale technical inconsistency in sampling and extraction technique. This high variance may have also been compounded by small sample sizes, particularly for the male krill. All of these factors could contribute to the observed range of age-pigment values. The high variance means that caution must be exercised in applying the pigment growth curves for ageing purposes, particularly for the older krill when the rate of pigment accumulation is much lower, causing the effects of high variance to be more pronounced.

Growth and pigment accumulation curves indicated that increase in both size and amount of age-pigment is rapid in early life, slowing significantly by 3 years of age. Similarly, Ju *et al.* (2001) found pigment accumulation rate slowed and then declined slightly at the end of experiments. This was attributed to reduced growth rate and reduced metabolism which occurs in older animals (Ikeda, 1985, Sheehy, 1992, Torres *et al.*, 1994). Another explanation is that the rate processes of pigment formation in post-mitotic neurons may be obscured by cellular turn-over of pigment that is occurring in mitotic cells, removing the 'waste deposits' of age-pigment. The technique used in this study would have extracted age-pigments from all tissues within a sample, mitotic and post-mitotic. It is unlikely that for animals the size of krill that it would be possible to extract only neural tissue, however, for vertebrates this is a distinct possibility.

It has also been suggested that a seasonal 'growth dilution effect' may occur where tissue mass (protein) is increasing rapidly compared to age-pigment accumulation, obscuring the real pigment accumulation rate (Ju *et al.*, 2001). The latter is unlikely for krill where the protein in the eye and eyestalk probably does not vary dramatically with somatic growth as an adult, although changes in body tissue have been shown to occur with shrinkage (McGaffin *et al.*, 2002). The wet weight of the krill eyes and eyestalks (WWE), however, had a positive relationship with age that was more highly correlated with age than carapace length. Eye diameter has been shown previously to be related to age, because the size is conserved during periods of body shrinkage, and increases when krill resume positive growth (Shin & Nicol, 2002). Additionally, WWE was not significantly affected by temperature, diet or stress in this study, implying that it might provide a more precise (if less accurate) ageing tool when using data from laboratory experiments to predict wild krill population demography; it also has the advantage of being a relatively simple measure.

The absolute relationship between age-pigment and body size over the entire size range of krill was difficult to assess, as no large krill were available for this study, either from the laboratory-reared populations or from the wild-caught, captive populations. Captive krill rarely, if ever, reach the maximum sizes observed in the field, even though they become reproductively mature and can live for several years (Ikeda and Thomas, 1987). In future studies, the techniques used in this study should be used to evaluate age-pigment quantities over the complete range of size classes of krill.

There have been different approaches to investigating age-pigments and their accumulation with time and relationship to metabolism. Histological methods have been applied widely and successfully to age many marine species: spider crabs (Hirche & Anger, 1988), prawns (Vila *et al.*, 2000), lobsters (O'Donovan & Tully, 1996), crayfish (Sheehy, 1990c; 1992; Belchier *et al.*, 1998), fish (Hammer, 1988; Vernet *et al.*, 1988; Hill & Womersley, 1993) molluscs (Clarke *et al.* 1990; Basova *et al.*, 2008), amphipods (Bluhm *et al.*, 2001a) and stomatopods (Kodama *et al.*, 2006). The histological approach, however, has not been of use for Antarctic krill because it has proved very difficult to detect age-pigment using this method (Sheehy, 1990b; Bluhm *et al.* 2001b; personal observation). Similarly to the Antarctic krill, it was found that the blue crab *Callinectes sapidus* was not

amenable to age determination using the histological approach (Harvey *et al.*, 2008). The alternative approach of using biochemical extraction was developed in response to the need to age the crab (Ju *et al.*, 1999, 2001, 2003), which is a commercially and ecologically important species (McMillen-Jackson & Bert, 2004).

Because the extraction method and the histological approach have not been applied successfully to the same populations there is some question over whether the two techniques are measuring the same pigments. It has been pointed out that there may be significant problems associated with using the extraction procedure over the histological approach (Sheehy, 2008). The first criticism is that the extracted fluorescent compounds remain unidentified and no correlation between extracted pigment and *in situ* age-pigment lipofuscin has yet been demonstrated (Sheehy, 1996; Porta, 2002). The incomplete characterisation of age-pigment is partly due to its heterogenous nature (Brunk & Terman, 2002). Sheehy (2008) puts forth an argument that the fluorescence of extracts may be attributable to other non-lipofuscin pigments. These include the derivatives of tryptophan, carotenoid, vitamin A, vitamin B6 and folic acid, pyridine nucleotides, flavins, proteins, and pteridines (Udenfriend 1962, 1969; Sheehy & Ettershank 1988; Sheehy & Roberts 1991; Ju *et al.*, 1999). Sheehy (1996, 2008) goes on to caution that because of this uncertainty the use of fluorescent extracts for age determination should be avoided. If any of these fluorescent compounds showed a relationship between accumulation and time, however, then they can be justifiably be termed an 'age-pigment'. That they are probably not lipofuscin as identified in histological studies, is only important for comparative purposes. The lack of characterisation does not discount the use of pigments for ageing purposes using either method (Harvey *et al.*, 2008). For the purpose of the present study, the hypotheses that pigments accumulate in krill and can be extracted, measured and correlated with age in known-age animals, was tested and satisfied. One of the most rigorous methods of validating ageing research is the use of known-age animals reared in conditions that reflect wild conditions (Campana, 2001). Because any ageing approach for commercially exploited species must be used with caution, it is vital that the technique be validated with precision estimates, and ideally be confirmed by other research (Harvey *et al.*, 2008).



The second criticism of the extraction method is that fluorescence maxima are dependent on tissue mass, (more tissue results in higher fluorescence measured). It has been asserted that there is a lack of evidence that standardising extracted pigments against cellular protein reliably takes into account tissue sample size differences, and where fresh weight might satisfy the same purpose (Crossland *et al.*, 1988; Sheehy, 2008). In the field of biochemistry, protein is routinely used as a primary method of standardisation for the determination of cellular constituents, enzymatic activities and specific synthesis processes (Harvey *et al.*, 2008). Cellular protein measures are also used to assess oxidative damage to proteins (Buss *et al.*, 1997), which may be pre-cursors to age-pigment formation. The accumulation of oxidized proteins is a characteristic feature of ageing cells (Grune *et al.*, 2001) and protein is a major constituent of age-pigment (Jolly, 2002). It is logical, therefore, to use a measure of protein, which is a 'primary cellular starting material', for standardisation (Harvey *et al.*, 2008). Using the fresh weight of dissected tissue for standardisation (eyes plus eyestalks for krill in this study), is confounded by the percentage of tissue that contains neither age pigment nor protein, but which would 'dilute' pigment estimates. Further, it has been postulated that apparent accumulation of age-pigment might be a function of increasing organ size, rather than increasing time, *per se* (Sheehy, 2008). This was clearly demonstrated not to be the case in the present study.

Thirdly, Sheehy (2008) expresses concern 'that high through-put of samples by using a biochemical technique over the laborious histological technique does not compensate for the lack of precision'. Whilst this is true enough, it appears that the histological technique cannot be applied to all species, and that an imprecise ageing method used with due care can augment conventional demographic information where there is an obvious need. Studies on age pigments in Antarctic krill have successfully drawn attention to the discrepancy between size and age, and this thesis has provided further evidence that age pigment accumulation might be a useful method to separate age classes. Where pigment studies lose out to conventional morphometrics is in the ease of application and in the standardisation of techniques. It will always be more difficult to measure age pigments than measure the length or weight of a krill; this explains why pigment-based ageing techniques have not been universally adopted for any species. Age pigments are a very useful way of determining what might be likely in terms of the

age structure of a population and this information can then be fed back into the interpretation of conventional morphometric studies.

## **Summary & Conclusions**

This study has demonstrated that nutrition, temperature and stress affect growth and mortality in captive Antarctic krill. Growth is integrally linked with metabolism and therefore nutrition, temperature and stress are also implicated in the formation and accumulation of metabolically produced pigments. It was demonstrated that these pigments could be biochemically extracted from eyes and eyestalks of krill and standardised against cellular protein. The pigments were found to accumulate with time in known-age krill, representing the first such calibration of age-pigment for this species. Temperature, nutrition and stress were also found to influence the accumulation of age-pigment. The model which was developed to predict age, based on the morphological characteristics of carapace length, wet weight of the eyes or age-pigments, demonstrated the increased value of age-pigment for this purpose, particularly when combined with the wet weight of the eyes. This information has the capacity to increase the body of knowledge about the demography of Antarctic krill, and reduce some of the uncertainty surrounding age estimation. Better age estimation of wild krill populations can lead to more accurate stock assessment, and this has implications for the management of the krill fishery. A well managed krill fishery is essential for the conservation of the Antarctic ecosystem.



## Acknowledgments

---

I'd like to acknowledge my supervisors for their guidance during this project. Dr George Jackson offered advice in a supervisory role during the conception and early stages of this project. Drs So Kawaguchi and Patti Virtue kindly took up supervisory roles later in the project when George was unable to continue to provide supervision. So and Patti provided me with valuable discussions and support to get me 'over-the-line'. Dr Steve Nicol, my supervisor from beginning to end, continued to believe in me and motivate me during the times when things got tough. Steve was always there to keep things in perspective and me on track. For this, Steve, I am eternally grateful.

The Australian Antarctic Division – Southern Ocean Ecosystems program supported the laboratory and field work. I would particularly like to thank Rob King, Paul Cramp, Luke Finley and Edwina Hollander for their assistance and friendship in sometimes uncomfortable laboratory conditions. Big thanks also to Debbie Lang who went above-and-beyond the call of duty on many occasions to support my laboratory activities. Dr Steve Candy assisted with statistical advice, demonstrations and helpful discussions.

I am indebted to the director and staff of Port of Nagoya Public Aquarium, who so generously supplied me with a number of krill that they had reared from eggs in the laboratory. In particular I would like to thank Mr Yasuo Hirano, Mr Tsuyoshi Matsuda and Dr Itaru Uchida (Director of the aquarium), and Dr So Kawaguchi who facilitated our contact.

I would also like to thank Drs Rodger Harvey and Se-Jong Ju from the University of Maryland Centre for Ecological Studies, for providing me with a protocol for the pigment extraction, and a great deal of supportive correspondence as I discovered how to do the analysis, and Dr Simon Wright and Rick van den Enden at the Australian Antarctic Division for helpful discussions about technical aspects of the analysis.

The thesis was greatly improved thanks to careful proof-reading by Edwina Hollander, Catherine Silcock, Klaus Meiners and my supervisors: Patti, So and Steve.

I am grateful to my friends and family for their support - particularly my mother Marilyn Raspin and my dear friend Catherine Silcock - for the many hours of childcare, encouraging discussions and meals they provided - without these fantastic women there simply wouldn't be a thesis.

I would like to dedicate this thesis to my beautiful children, Matthew and Isabelle; that they might have a brighter future because of it.

This research was supported by a University of Tasmania/Australian Antarctic Division scholarship 2000-2004.

## Literature cited

---

- Alonzo, F., Virtue, P., Nicol, S. & Nichols, P. 2005 Lipid as trophic markers in Antarctic krill. II. Lipid composition of the body and digestive gland of *Euphausia superba* in controlled conditions. *Mar. Ecol. Prog. Ser.* 296: 65-79.
- Alonzo, S.H. & Mangel, M. 2001 Survival strategies and growth of krill: avoiding predators in space and time. *Mar. Ecol. Prog. Ser.* 209: 203-217.
- Alonzo, S.H. & Mangel, M. 2002 Effects of social behaviour on survival and growth of krill: important, but how relevant? *Mar. Ecol. Prog. Ser.* 244: 309-310.
- Alonzo, S.H., Switzer, P.V. & Mangel, M. 2003 Ecological Games in Space and Time: The Distribution and Abundance of Antarctic Krill and Penguins *Ecology*. 84(6): 1598–1607.
- Aseev, Y.P. 1983 Size structure and longevity of krill population from the Indian Ocean sector of the Antarctic. In: Lyubimova, T.G. *Antarctic krill. Distribution pattern and environment*. (In Russian). Sb. Nauch. Tr. VNIRO pp.103-110.
- Atkinson, A. & Snyder, R. 1997 Krill-copepod interactions at South Georgia, Antarctica, I - Omnivory by *Euphausia superba*. *Mar. Ecol. Prog. Ser.* 160: 63-76.
- Atkinson, A., Meyer, B., Stübing, D., Hagen, W., Schmidt, K. & Bathmann, U. 2002 Feeding and energy budgets of Antarctic krill *Euphausia superba* at the onset of winter – II. Juveniles and adults. *Limnol. Oceanogr.* 47(4): 953-966.
- Atkinson, A., Siegel, V., Pakhomov, E. & Rothery, P. 2004 Long-term decline in krill stock and increase in salps within the Southern Ocean. *Nature*. 432: 100-103.
- Atkinson, A., Shreeve, R.S., Hirst, A.G., Rothery, P., Tarling, G.A., Pond, D.W. Korb, R.E., Murphy, E.J. & Watkins, J.L. 2006 Natural growth rates in Antarctic krill (*Euphausia superba*): II. Predictive models based on food, temperature, body length, sex, and maturity stage. *Limnol. Oceanogr.* 51(2): 973-987.
- Atkinson, A., Siegel, V., Pakhomov, E. A., Rothery, P., Loeb, V. Ross, R. M., Quetin, L. B., Schmidt, K., Fretwell, P., Murphy, E. J., Tarling, G. A. & Fleming A. H. 2008 Oceanic circumpolar habitats of Antarctic krill. *Mar. Ecol. Prog. Ser.* In Press.

- Baker, A. de C., Boden, B.P. & Brinton, E. 1990 *A practical guide to the Euphausiids of the world*. British Museum - Natural History, London. 96 pp.
- Bargmann, H.E. 1945 The development and life history of adolescent and adult krill, *Euphausia superba*. *Discovery Rep.* 23: 103-176.
- Basova, L., Begum, S., Strahl, J., Philipp, E., Heilmayer, O., Sukhotin, A., Brey T. & Abele D. 2008 Longevity of *Arctica islandica* in different populations: Energy metabolism, antioxidant capacity and age pigment accumulation. *Comp. Biochem. Physiol. A: Mol. & Int. Physiol.* 151 (1) S1: S24.
- Beddington, J.R. & May, R.M. 1982 The harvesting of interacting species in a natural ecosystem. *Sci. Am.* 247: 42 - 49.
- Belchier, M., Shelton, P.M.J. & Chapman, C.J. 1994 The identification and measurement of fluorescent age-pigment abundance in the brain of a crustacean (*Nephrops norvegicus*) by confocal microscopy. *Comp. Biochem. Physiol. B.* 108B (2) 157-164.
- Belchier, M., Edsman, L., Sheehy, M. & Shelton P. 1998 Estimating age and growth in long-lived temperate freshwater crayfish using lipofuscin. *Freshwater Biol.* 39 (3): 439-446.
- Bergmann, M., Taylor A.C. & Moore, P.G. 2001 Physiological stress in decapod crustaceans (*Munida rugosa* and *Liocarcinus depurator*) discarded in the Clyde Nephrops fishery. *J. Exp. Mar. Biol. Ecol.* 259 (2): 215-229.
- Berman, M.S., McVey A.L. & Ettershank G. 1989 Age determination of Antarctic krill using fluorescence and image analysis of size. *Polar Biology.* 9 (4) 267-271.
- Bindoff, N. L., Rosenberg M.A., & Wamer, M. J. 2000 On the circulation of the waters over the Antarctic continental slope and rise between 80 to 150°E. *Deep-Sea Res. II: Topical Studies Oceanogr.* 47(12-13): 2299-2326.
- Bluhm, B.A., Brey, T. & Klages, M. 2001a The autofluorescent age-pigment lipofuscin: key to age, growth and productivity of the Antarctic amphipod *Waldeckia obesa* (Chevreux, 1905). *J. Exp. Mar. Biol. Ecol.* 258 (2): 215-235.
- Bluhm, B.A., Brey, T., Klages, M. & Amtz, W.E. 2001b Occurrence of the autofluorescent pigment, lipofuscin, in polar crustaceans and its potential as an age marker. *Polar Biol.* 24 (9): 642-649.
- Brierley, A.S., Fernandes, P.G., Brandon, M.A., Armstrong, F., Millard, N.W., McPhail, S.D., Stevenson, P., Pebody, M., Perrett, J., Squires, M., Bone, D.G. & Griffiths, G. 2002 Antarctic Krill Under Sea Ice:

Elevated Abundance in a Narrow Band Just South of Ice Edge. *Science*. 295 (5561): 1890 – 1892.

Brunk, U.T. & Terman, A. 2002 Lipofuscin: mechanisms of age-related accumulation and influence on cell functions. *Free Radic. Biol. Med.* 33: 611-619.

Buchholz, F. 1982 Drach's molt staging system adapted for euphausiids. *Mar. Biol.* 66: 301-305.

Buchholz, F. 2003. Experiments on the physiology of Southern and Northern krill, *Euphausia superba* and *Meganyctiphanes norvegica*, with emphasis on moult and growth: A review. *Mar. Freshwater Behav. Physiol.* 36(4): 229-247.

Buchholz, F. 1991 Moulting cycle and growth of Antarctic krill *Euphausia superba* in the laboratory. *Mar. Ecol. Prog. Ser.* 69 (3): 217-229.

Buchholz, F., Watkins, J.L., Priddle, J., Morris, D.J., Ricketts, C. 1996 Moulting in relation to some aspects of reproduction and growth in swarms of Antarctic krill, *Euphausia superba*. *Mar. Biol.* 127(2): 201-208.

Buss, H. Chan, T.P., Sluis, K.B., Domigan, N.M. & Winterbourn, C.C. 1997 Protein carbonyl measurement by a sensitive ELISA method. *Free. Radic. Biol. Med.* 23: 361-366.

Camougrand, N. & Rigoulet, M. 2001 Aging and oxidative stress: studies of some genes involved both in aging and in response to oxidative stress. *Resp. Physiol.* 128: 393-401.

Campana, S.E. 2001 Accuracy, precision and quality control in age determination, including a review of the use and abuse of age validation methods. *J. Fish. Biol.* 59: 197-242.

Candy, S.G. 1991 Modeling insect phenology using ordinal regression and continuation ratio models. *Environmental Entomology*. 20: 190-195.

Candy, S.G. 2003 Predicting time to peak occurrence of insect life-stages using regression models calibrated from stage-frequency data and ancillary stage-mortality data. *Agricultural and Forest Entomology*. 5: 1-7.

Candy, S.G. & Kawaguchi, S. 2006 Modeling growth of Antarctic krill. II. Novel approach to describing the growth trajectory. *Mar. Ecol. Prog. Ser.* 306: 17-30.

Carlisle, D.B. & Knowles, F.B. 1959 Growth, moulting, development and metabolism. In: *Endocrine control in crustaceans*. Cambridge University Press, London. 120 pp.



- Castro, M., Encarnação, P. & Tully, O. 2002 The effect of dietary antioxidants on lipofuscin accumulation in the crustacean brain. *J. Exp. Mar. Biol. Ecol.* 269 (1): 53-64.
- Chekunova, V.I. & Rynkova, T.I. 1974 Energy requirements of the Antarctic crustacean *Euphausia superba* Dana. *Oceanology.* 14: 434-440.
- Chiba, S. 2007 A review of ecological and evolutionary studies on hermaphroditic decapod crustaceans. *Plankton Benthos Res.* 2 (3): 107-119.
- Clarke, A. & Morris, D.J. 1983 Towards an energy budget for krill: the physiology and biochemistry of *Euphausia superba* Dana. *Polar Biol.* 2: 69-86.
- Clarke, A., Kendall, M. A. Gore, D. J. 1990 The accumulation of fluorescent age pigments in the trochid gastropod *Monodonta lineata*. *J. Exp. Mar. Biol. Ecol.* 144: 185-204.
- Commission for the Conservation of Antarctic Marine Living Resources (CCAMLR) 2006 Stock assessment and management models website <http://www.ccamlr.org/pu/E/sc/fish-monit/fm-stock-assess.htm> cited Feb 20th 2006.
- Constable, A.J. & de la Mare, W.K. 1996 A generalised model for evaluating yield and the long-term status of fish stocks under conditions of uncertainty. *CCAMLR Sci* 3: 31-54.
- Constable, A.J., de la Mare, W.K., Agnew, D.J. Everson, I. & Miller, D. 2000 Managing fisheries to conserve the Antarctic marine ecosystem: practical implementation of the Convention on the Conservation of Antarctic Marine Living Resources (CCAMLR). *ICES J Mar. Sci.* 57: 778-791.
- CORDIS: FAIR 2008 Agriculture and fisheries website [http://cordis.europa.eu/data/PROJ\\_FAIR/ACTIONeqDndSESSIONeq5640200595ndDOCeql0ndTBLeqEN\\_PROJ.htm](http://cordis.europa.eu/data/PROJ_FAIR/ACTIONeqDndSESSIONeq5640200595ndDOCeql0ndTBLeqEN_PROJ.htm) cited Dec 19<sup>th</sup> 2008.
- Cripps, G.C. & Atkinson, A. 2000 Fatty acid composition as an indicator of carnivory in Antarctic krill *Euphausia superba*. *Can. J. Fish. Aquat. Sci.* 57 (S3): 31-37.
- Cripps, G.C. & Priddle, J. 1991 Hydrocarbons in the Antarctic marine environment. *Ant. Sci.* 3 (3): 233-250.
- Crossland, C.J., Denby, G., Phillips, B.F. & Brown, R. 1988 The use of fluorescent pigment (lipofuscin) for ageing western rock lobster (*Panulirus cygnus*) and scampi (*Metanephrops andamanicus*): a preliminary assessment. CSIRO Marine Laboratories Report 195. Commonwealth Scientific and Industrial Research Organisation, Hobart.

- Daly, K.L. 1990 Overwintering development, growth, and feeding of larval *Euphausia superba* in the Antarctic marginal ice zone. *Limnol. Oceanogr.* 35 (7): 1564-1576.
- Daly, K.L. & Macaulay, M.C. 1991 Influence of physical and biological mesoscale dynamics on the seasonal distribution and behaviour of *Euphausia superba* in the Antarctic marginal ice zone. *Mar. Ecol. Prog. Ser.* 79: 37-66.
- Davis, B.O., Anderson, G.L. & Dusenberger, D.B. 1982 Total luminescent spectroscopy of fluorescent changes during aging in *Caenorhabditis elegans*. *Biochem.* 21: 4089-4095.
- Dayan, D., Abrahami, I., Buchner, A., Gorsky, M. & Chimovitz, N. 1988 Lipid pigment (lipofuscin) in human peroral muscles with aging. *Exp. Geront.* 23: 97-102.
- Dictionary.com. *Dictionary.com Unabridged (v 1.1)*. Random House, Inc. <http://dictionary.reference.com/browse/krill> (accessed: June 24, 2008).
- Eldred, G.E., Miller, G.V., Stark, W.S. & Feeney-Burns, L. 1982 Lipofuscin: resolution of discrepant fluorescence data. *Science.* 216: 757-759.
- El-Sayed, S.Z. 1994 History, organization and accomplishments of the BIOMASS program. In: *Southern Ocean ecology: the BIOMASS perspective*. Ed: El-Sayed, S.Z. Cambridge University Press, Cambridge, U.K. pp:1-8.
- Ettershank, G. 1983a Age structure and cyclical annual size change in the Antarctic krill, *Euphausia superba* Dana. *Polar Biol.* 2: 189-193.
- Ettershank, G. 1983b The age structure of a population of the Antarctic krill *Euphausia superba* Dana. Seminar and report of the krill ecology group, Bremerhaven (FRG), 12 May 1983.
- Ettershank, G. 1984a A new approach to the assessment of longevity in Antarctic krill *Euphausia superba*. *J. Crust. Biol.* 4 (1): 295-305.
- Ettershank, G. 1984b Methodology for age determination of Antarctic krill using the age pigment lipofuscin. *BIOMASS Handbook* 26. Cambridge, U.K. 14 pp.
- Ettershank, G. 1985 Population age structure in males and juveniles of the Antarctic krill, *Euphausia superba* Dana. *Polar Biol.* 4: 199-201.
- Everson, I. 2000 Introducing Krill. In: Everson I. (Ed.) *Krill: Biology, Ecology & Fisheries*. Blackwell Science Ltd, Oxford. pp 1-7.
- Everson, I. 2000 The Southern Ocean. In: Everson I. (Ed.) *Krill: Biology, Ecology & Fisheries*. Blackwell Science Ltd, Oxford. pp 63-79.

- Everson, I. & de la Mare, W.K. 1996 Some thoughts on precautionary measures for the krill fishery. *CCAMLR Sci.* 3: 1-11.
- Fabry, V. J., Seibel, B. A., Feely, R. A., and Orr, J. C. 2008. Impacts of ocean acidification on marine fauna and ecosystem processes. *ICES J. Mar. Sci.*, 65: 414-432.
- Fach, B.A., Hofmann, E.E. & Murphy, E.J. 2002 Modeling studies of Antarctic krill *Euphausia superba* during transport across the Scotia Sea. *Mar. Ecol. Prog. Ser.* 231: 187-202.
- Farber-Lorda, J. 1991 Multivariate approach to the morphological and biochemical differentiation of Antarctic krill (*Euphausia superba* and *Thysanoessa macrura*). *Deep Sea Res.* 38(7): 771-779.
- Fattoretti, P., Bertoni-Freddari, C., Casoli, T., Di Stefano, G., Solazzi M. & Corvi E. Morphometry of age pigment (lipofuscin) and of ceroid pigment deposits associated with vitamin E deficiency. *Arch. Gerontol. Ger.* 34 (3): 263-268.
- Fevolden, S.E. 1979 Investigations on krill (Euphausiacea) sampled during the Norwegian Antarctic Research Expedition, 1976-1977. *Sarsia.* 64: 189-198.
- Finch, C.E. 1990 Longevity, senescence and the genome. Chicago University Press, Chicago, U.S.A.
- Finkel, T. & Holbrook, N.J. 2000 Oxidants, oxidative stress and the biology of ageing. *Nature.* 408:239-247.
- Fisheries and Agriculture Organisation of the United Nations 2006 Fisheries Global Information System (FIGIS) website  
<http://www.fao.org/figis/servlet/species?fid=3393> cited Feb 7 2006.
- Fletcher, B.L., Dillard, C.J., Tappel, A.L. 1973 Measurement of fluorescent lipid peroxidation products in biological systems and tissues. *Anal. Biochem.* 52:1-9.
- Fraser, F.C. 1936 On the development and distribution of the young stages of krill (*Euphausia superba*). *Discovery Rep.* 14: 1-192.
- Fraser, W.R. & Hofmann, E. 2003 A predator's perspective on causal links between climate change, physical forcing and ecosystem response. *Mar. Ecol. Prog. Ser.* 265: 1-15.
- Girven, R.J. Gauldie, R.W., Czochanska, Z. & Woolhouse, A.D. 1993 A test of the lipofuscin technique of age estimation in fish. *J. Appl. Ichthyol.* 9:82-88.

- Goebel, M.E., Lipsky J.D., Reiss, C.S. & Loeb V.J. 2007 Using carapace measurements to determine the sex of Antarctic krill, *Euphausia superba*. *Polar Biol.* 30:307–315.
- Gómez-Guitérrez, J., Peterson, W.T., de Robertis, A. & Brodeur, R.D. 2003 Mass mortality of krill caused by parasitoid ciliates. *Science* 301: 339.
- Green, J. 1956 Growth, size and reproduction in *Daphnia* (Crustacea: Cladocera). *Proc. Zool. Soc. Lond.* 126: 173-204.
- Grune, T., Shringarpure, R., Sitte N. & Davies K. 2001 Age-Related Changes in Protein Oxidation and Proteolysis in Mammalian Cells. *J. Geront. Ser. A: Biol. Sci. Med. Sci.* 56B: 459-467.
- Grune, T., Merkera, K., Jung, T., Sitte N., & Davies K.J.A. 2005 Protein oxidation and degradation during postmitotic senescence. *Free Rad. Biol. Med.* 39 (9):1208-1215.
- Haberman, K.L., Quetin L.B & Ross R.M. 2003 Diet of the Antarctic krill (*Euphausia superba* Dana): I. Comparisons of grazing on *Phaeocystis antarctica* (Karsten) and *Thalassiosira antarctica* (Comber). *J. Exp. Mar. Biol. Ecol.* 283 (1-2): 79-95.
- Hammer, C. 1988 Accumulation of fluorescent age pigments in embryos and larvae of pike, *Esox lucius* (Esocidae, Teleostei) at different incubation temperatures. *Env. Biol. Fish.* 22(2): 91-99.
- Hamner, W.M. & Hamner, P.P. 2000 Behaviour of Antarctic krill (*Euphausia superba*): schooling, foraging and anti-predatory behaviour. *Can. J. Fish. Aquat. Sci.* 57 (S3): 192-202.
- Harman, D. 1957 Ageing: a theory based on free radical and radiation chemistry. *J. Gerontol.* 2: 298-300.
- Harvey, H.R., Secor, D.H. & Ju, S.-J. 2008 The use of extractable lipofuscin for age determination of crustaceans: Reply to Sheehy (2008). *Mar. Ecol. Prog. Ser.* 353: 307-311.
- Harvey, H.R., Ju, S.-J., Son, S.K., Feinberg, L.R., Shaw, C.T. & Peterson, W.T. In press. The biochemical estimation of age in Euphausiids: laboratory calibration and field comparisons. *Deep Sea Research II: special issue on krill biology and ecology*.
- Haywood, G.J. & Burns, C.W. 2003 Growth of *Nyctiphanes* (Euphausiacea) on different diets. *J. Exp. Mar. Biol. Ecol.* 289 (1): 139-151.
- Hill, K.T. & Womersley, C.Z. 1993 Interactive effects of some environmental and physiological variables on fluorescent age pigment accumulation in brain and heart tissues of an aquatic poikilotherm. *Env. Biol. Fish.* 37 (4): 397-405.

- Hirano, Y. & Matsuda T. 2003 Antarctic krill breeding facilities at Port of Nagoya Public Aquarium. *Mar. Fresh. Behav. Physiol. Spec. Iss: Understanding living krill for improved management and stock assessment*. 36 (4): 249-258.
- Hirano, Y., Matsuda, T. & Kawaguchi, S. 2003 Breeding Antarctic krill in captivity. *Mar. Freshwater Behav. Physiol. Spec. Iss: Understanding living krill for improved management and stock assessment*. 36 (4): 259-269.
- Hill, K.T. & Womersley, C. 1991 Critical aspects of fluorescent age-pigment methodologies: modification for accurate analysis and age assessments in aquatic organisms. *Mar. Biol.* 109: 1-11.
- Hill, H.J., Trathan, P.N., Croxall, J.P. & Watkins, J.L. 1996 A comparison of Antarctic krill *Euphausia superba* caught by nets and taken by Macaroni penguins *Eudyptes chrysolophus*: evidence for selection? *Mar. Ecol. Prog. Ser.* 140: 1-11.
- Hirano, Y. & Matsuda, T. 2003 Antarctic krill breeding facilities at Port of Nagoya Public Aquarium. *Mar. Fresh. Behav. Physiol.* 36 (4): 249-258.
- Hirano, Y., Matsuda, T. & Kawaguchi, S. 2003 Breeding Antarctic krill in captivity. *Mar. Fresh. Behav. Physiol.* 36 (4): 259-269.
- Hirche, H.J. 1983 Excretion and respiration of the Antarctic krill *Euphausia superba*. *Polar Biol.* 1: 205-209.
- Hirche, H.J. 1984 Temperature and metabolism of plankton – I. Respiration of Antarctic zooplankton at different temperatures with a comparison of Antarctic and Nordic krill. *J. Comp. Biochem. Physiol. A.* 77 (2):361-368.
- Hoenig, J.M. 1983 Empirical use of longevity data to estimate mortality rates. *Fish. Bull.* 82 (1): 898-903.
- Hofmann, E.E., Klinck, J.M., Costa, D.P., Daly, K.L., Torres, J.J. and Fraser, W.R. 2002. U.S. Southern Ocean Global Ocean Ecosystems Dynamics Program. *Oceanography* 15(2) 64-74.
- Horst, M. & Freeman, J.A. 1993 (Eds.) *The Crustacean integument – morphology and biochemistry*. CRC Press Inc. Boca Raton, Florida.
- Iguchi, N. & Ikeda, T. 2005 Effects of temperature on metabolism and growth efficiency of *Thysanoessa longipes* (Crustacea: Euphausiacea) in the Japan Sea. *J. Plank. Res.* 27 (1): 1-10.
- Ikeda, T. 1985 Life history of Antarctic krill *Euphausia superba*: A new look from an experimental approach. *Bull. Mar. Sci.* 37: 599-608.

- Ikeda, T. & Bruce, B. 1986 Metabolic activity and elemental composition of krill and other zooplankton from Prydz Bay, Antarctica, during early summer (November-December). *Mar. Biol.* 92: 545-555.
- Ikeda, T. & Dixon, P.G. 1982 Body shrinkage as a possible over-wintering mechanism of the Antarctic krill *Euphausia superba* Dana. *J. Exp. Mar. Biol. Ecol.* 63: 143-151.
- Ikeda, T. & Hing Fay, E. 1981 Metabolic activity of zooplankton from the Antarctic ocean. *Aust. J. Mar. Freshw. Res.* 32: 921-930.
- Ikeda, T. & Mitchell, A.W. 1982 Oxygen uptake, ammonia excretion and phosphate excretion by krill and other Antarctic zooplankton in relation to their body size and chemical composition. *Mar. Biol.* 71: 283-298.
- Ikeda, T. & Thomas, P.G. 1987a Moulting interval and growth of juvenile Antarctic krill (*Euphausia superba*) fed different concentrations of the diatom *Phaeodactylum tricornutum* in the laboratory. *Polar Biol.* 7: 339-343.
- Ikeda T. & Thomas P.G. 1987b Longevity of the Antarctic krill (*Euphausia superba* Dana) based on a laboratory experiment. *Proc. Natl. Inst. Polar Res. Symp. Polar Biol.* 1: 56-62.
- Ikeda, T., Dixon, P. & Kirkwood, J. 1985 Laboratory observations on moulting, growth and maturation in Antarctic krill (*Euphausia superba* Dana). *Polar Biol.* 4: 1-18.
- Ishii, H., Omori, M., Maeda, M. & Watanabe, Y. 1987 Metabolic rates and elemental composition of Antarctic krill *Euphausia superba* Dana. *Polar Biol.* 7: 379-382.
- Ivanov, B.G. 1970 On the biology of the Antarctic krill (*Euphausia superba* Dana). *Mar. Biol.* 7: 340-351.
- Johnson, M. J. & Tarling G.A. 2008 Influence of individual state on swimming capacity and behaviour of Antarctic krill *Euphausia superba*. *Mar. Ecol. Prog. Ser.* 366: 99-110.
- Jolly, R.D., Palmer, D.N., Dalefield, R.R. The analytical approach to the nature of lipofuscin (age pigment). *Arch. Geront. Ger.* 34 (3): 205-217.
- Ju, S.-J., Secor, D.H. & Harvey, H.R. 1999 Use of extractable lipofuscin for age determination of blue crab *Callinectes sapidus*. *Mar. Ecol. Prog. Ser.* 185: 171-179.
- Ju, S.-J., Secor D.H. & Harvey, H.R. 2001 Growth rate variability and lipofuscin accumulation rates in the blue crab *Callinectes sapidus*. *Mar Ecol. Prog. Ser.* 224: 197-205.

- Ju, S.-J., Secor, D.H., Harvey, H.R. 2003 Demographic assessment of the blue crab (*Callinectes sapidus*) in Chesapeake Bay using extractable lipofuscins as age markers. *Fish. Bull.* 101: 312-320.
- Kanda, K., Takagi, K. & Seki, Y. 1982 Movement of the larger swarms of Antarctic krill *Euphausia superba* off Enderby Land during 1976-77 season. *J. Tokyo Univ. Fish.* 68: 24-42.
- Katz, M. 2002 Potential reversibility of lipofuscin accumulation. *Arch. Gerontol. Ger.* 34 (3): 311-317.
- Katz, M.L., White, H.A., Gao, C.L., Roth, G.S., Knapka, J.J. & Ingram, D.K. 2003 Dietary restriction slows age pigment accumulation in the retinal pigment epithelium. *Inv. Ophthalmol. Vis. Sci.* 34: 3297-3302.
- Kawaguchi, K., Matsuda, O., Ishikawa, S. & Naito, Y. 1986 The overwintering strategy of Antarctic krill (*Euphausia superba*) under the coastal fast ice off the Ongul Island in Lutzow-Holm bay, Antarctica. *Mem. Nat. Inst. Polar Res. Tokyo* (Spec. Iss.) 44: 67-85.
- Kawaguchi, S. & Satake M. 1994. Relationship between recruitment of the Antarctic krill and the degree of ice cover near the South Shetland Islands. *Fish. Sci.*, 60: 123-124.
- Kawaguchi, S. & Toda, T. 1997 Discovery of ciliates reproducing in the gut of Antarctic krill. *Polar Biol.* 18 (2): 158-160.
- Kawaguchi, S., Candy, S.G., King, R., Naganobu, M. & Nicol, S. 2006 Modeling growth of Antarctic krill. I. Growth trends with sex, length, season, and region. *Mar. Ecol. Prog. Ser.* 306: 1-15.
- Kils, U. 1979 The swimming behaviour, swimming performance and energy balance of Antarctic krill, *Euphausia superba*. *Ber. Inst. Meereskunde Univ. Kiel* 65: 1-71.
- Kils, U. 1981 Swimming behaviour, swimming performance and energy balance of Antarctic krill, *Euphausia superba*. *BIOMASS Sci, Ser.* 3: 122pp.
- Kils, U. 1982 The swimming behaviour, swimming performance and energy balance of Antarctic krill, *Euphausia superba*. *BIOMASS Sci. Ser.* 3 122 pp.
- Kils, U. 1995 Wikipedia website  
[http://en.wikipedia.org/wiki/User:Kils/Antarctic\\_krill/frozen](http://en.wikipedia.org/wiki/User:Kils/Antarctic_krill/frozen)  
 Cited 10 Feb 2006.
- King, R., Nicol, S., Cramp, P. & Swadling, K.M. 2003 Krill maintenance and experimentation at the Australian Antarctic Division. *Mar. Fresh. Behav. Physiol.* 36(4):271-283.

- Kirkwood, J.M. 1982 *A guide to the Euphausiacea of the Southern Ocean. ANARE research notes 1*. Information services section, Australian Antarctic Division, Hobart, Australia. pp: 34-39.
- Kirkwood, T.B.L. 1977 Evolution of ageing. *Nature*. 270: 301-304.
- Kirkwood, T.B.L. & Austad, S.N. 2000 Why do we age? *Nature*. 408:233-238.
- Kock, K.-H., Reid, K., Croxall, J. & Nicol, S. 2007 Fisheries in the Southern Ocean: an ecosystem approach. *Phil. Trans. R. Soc. B* 362: 2333-2349.
- Knox, G.A. 1993 *Biology of the Southern Ocean*. CRC press. Boca Raton, Florida. 561 pp.
- Kodama, K., Shiraishi, H., Morita, M. & Horiguchi, T. 2006 Verification of lipofuscin-based crustacean ageing: seasonality of lipofuscin accumulation in the stomatopod *Oratosquilla oratoria* in relation to water temperature. *Mar. Biol.* 150: 131-140.
- Kruk, P. & Enesco, H.E. 1981  $\alpha$ -Tocopherol reduces fluorescent age pigment levels in heart and brain of young mice. *Cell. Mol. Life Sci.* 37 (12): 1301-1302.
- Lahdes, E.O. 1995. Acute thermal tolerance of two Antarctic copepods, *Calanoides acutus* and *Calanus propinquus*. - *J. Therm. Biol.* 20: 75-78.
- Lehane, M.J. & Mail, T.S. 1985 Determining the age of adult male and female *Glossina morsitans morsitans* using a new technique. *Ecol. Entomol.* 10: 219-224.
- Leibovitz, B.E. & Siegel, B.V. 1980 Aspects of free radical reactions in biological systems: aging. *J. Geront.* 35: 45-56.
- Lim, C., Akob, H., Brown C.L. & Hahn, K. 1997 Growth response and fatty acid composition of juvenile *Penaeus vannamei* fed different sources of dietary lipid. *Aquaculture. Fish Nutrition and Feeding Proceedings of the Sixth International Symposium on Feeding and Nutrition in Fish*. 151 (1-4): 143-153.
- Macauley, M.C., English, T.S. & Mathisen, O.E. 1984 Acoustic characterisation of swarms of Antarctic krill (*Euphausia superba*) from Elephant Island and Bransfield Strait. *J. Crust. Biol.* 4 (Special Issue): 16-44.
- Macdonald, P.D.M. & Green, P.E.J. (1985). Mix 2.2: an interactive program for fitting mixtures of distributions. Ichthus Data Systems, Hamilton, Ontario.



- Macdonald, P.D.M. & Pitcher, T.J. 1979 Age groups from size frequency data: a versatile and efficient method of analysing distribution mixtures. *J. Fish. Res. Bd. Can.* 36: 987-1001.
- Macintosh, N.A. 1972 Life-cycle of Antarctic krill in relation to the ice conditions. *Discovery Rep.* 34: 1-94.
- Mail, T.S., Chadwick, J. & Lehane, M.J. 1983 Determining the age of adults of *Stomoxys calcitrans* (L.) (Diptera: Muscidae). *Bull. Ent. Res.* 73: 501-525.
- Makarov, R.R. 1975 A study of repeated maturation of female euphausiids (Eucaridea, Euphausiacea). *Zool. Zh.* 54: 670-681.
- Marr, J. W. S. 1962 The natural history and geography of the Antarctic krill (*Euphausia superba*). *Discovery Rep.* 32: 33-464.
- Marschall, H.P. 1988 The overwintering strategy of the Antarctic krill under the pack-ice of the Weddell Sea. *Polar Biol.* 9: 129-135.
- Marschall, H.P. & Hirche, H.J. 1984 Development of eggs and nauplii of *Euphausia superba*. *Polar Biol.* 2 (4): 240-250.
- Martin, D. L., Ross, R. M., Quetin, L. B. and Murray, A. E. 2006 Molecular approach (PCR-DGGE) to diet analysis in young Antarctic krill *Euphausia superba*. *Mar Ecol Prog Ser.* 319: 155-165.
- Mauchline, J. 1980 Measurement of body length of *Euphausia superba* Dana. *BIOMASS Handbook No. 4*, 9 pp.
- Mauchline, J. 1985 Growth in Mysids and Euphausiids. In: Wenner, A.M. (Ed.) *Crustacean issues 3: factors in adult growth*. A.A. Balkema, Rotterdam, The Netherlands, CRC Press, 365pp.
- Mauchline, J. & Fisher, L.R. 1969 The Biology of Euphausiids. In: Russell, F.S. & Yonge, M. (Eds.) *Advances in Marine Biology – Volume 7*. Academic Press Inc., London. pp 1- 454.
- Mayer, G. 2006 Structure and development of onychophoran eyes: What is the ancestral visual organ in arthropods? *Arth. Struct. Dev.* 35 (4): 231-245.
- McArthur, M.C. & Sohal, R.S. 1982 Relationship between metabolic rate, aging, lipid peroxidation, and fluorescent age pigment in milkweed bug, *Oncopeltus fasciatus* (Hemiptera). *Gerontol.* 37 (30): 268-274.
- McMillen-Jackson, A. L. & Bert T. M., 2004 Mitochondrial DNA variation and population genetic structure of the blue crab *Callinectes sapidus* in the eastern United States. *Mar. Biol.* 145 (4): 769-777.

- Medina, A., Vila, Y., Megina, C., Sobrino, I & Ramos F. 2000 A histological study of the age-pigment, lipofuscin, in dendrobranchiate shrimp brains. *J. Crust. Biol.* 20 (3): 423-430.
- Microsoft Corporation. 1985-2001 Microsoft Office Excel 2002 SP3.
- Miller, D.G.M. & Agnew, D. 2000 Management of krill fisheries in the Southern Ocean. In: Everson I. (Ed.) *Krill: Biology, Ecology & Fisheries*. (Ed) Blackwell Science Ltd, Oxford. pp 301-337.
- Miller, D.G.M. & Hampton, I. 1989 *Biology and ecology of the Antarctic krill* (*Euphausia superba* Dana): a review. BIOMASS Sci. Ser. 9. SCAR & SCOR, Scott Polar Research Institute. Cambridge, England. 166 pp.
- McClatchie, S. 1988 Food-limited growth of *Euphausia superba* in Admiralty Bay, South Shetland Islands, Antarctica. *Continental Shelf Res.* 8 (4): 329-345.
- McClatchie, S., Rakusa-Suszczewski, S., & Filcek, K. 1991 Seasonal growth and mortality of *Euphausia superba* in Admiralty Bay, South Shetland Islands, Antarctica. *ICES J. Mar. Sci.* 48: 335-342.
- McCord, J.M. & Day, E.D. 1978 Superoxide-dependent production of hydroxyl radical catalyzed by iron-EDTA complex. *FEBS Lett.* 86(1): 139-142.
- McGaffin, A.F., Nicol, S. & Ritz, D. 2002 Changes in muscle tissue of shrinking Antarctic krill. *Polar Biol.* 25: 180-186.
- McWhinnie, M.A. & Marciniak, P. 1964 Temperature responses and tissue respiration in Antarctic crustacea with particular reference to krill, *Euphausia superba*. In: Lee, M.O. (Ed.) *Biology of Antarctic Seas*. *Antarct. Res. Ser.* 4: 63-72.
- McWhinnie, M.A., Denys, C.J. & Parkin, R.K. 1979 Biological investigations of *Euphausia superba*. *Antarct. J. U.S.* 15: 163-164.
- Meyer, B. & Oetl, B. 2005 Effects of short-term starvation on composition and metabolism of larval Antarctic krill *Euphausia superba*. *Mar. Ecol. Prog. Ser.* 292: 263-270.
- Meyer, B., Atkinson, A., Stübing, D., Oetl, B., Hagen, W. & Bathmann, U.V. 2002 Feeding and energy budgets of Antarctic krill *Euphausia superba* at the onset of winter- I. Furcilia III larvae. *Limnol. Oceanograph.* 47 (4): 943-952.
- Miller, A.K. & Trivelpiece W.Z. 2007 Cycles of *Euphausia superba* recruitment evident in the diet of Pygoscelid penguins and net trawls in the South Shetland Islands, Antarctica. *Polar Biol.* 30: 1615-1623.

- Miller, D.G.M. & Hampton, I. 1989 *Biomass Scientific Series, No. 9: Biology and ecology of the Antarctic krill (Euphausia superba Dana): a review*. SCAR and SCOR, Scott Polar Research Institute, Cambridge, England. 166 pp.
- Molina Grimal, E., Robles Medinal, A., Giménez Giménez A. & Ibáñez González, M. J. 1996 Gram-scale purification of eicosapentaenoic acid (EPA, 20:5n-3) from wet *Phaeodactylum tricornutum* UTEX 640 biomass. *J. Appl. Phycology*. 8 (4-5), 359-367.
- Morris, D.J., Ward, P. & Clarke, A. 1983 Some aspects of feeding in the Antarctic krill, *Euphausia superba*. *Polar Biol.* 2 (1): 21-26.
- Murano, M., Segawa, S. & Kato, M. 1979 Moults and growth of the Antarctic krill in the laboratory. *Trans. Tokyo. Univ. Fish.* 3: 99-106.
- Murphy, E.J., Watkins, J.L., Reid, K., Trathan, P.N., Everson, I., Croxall, J.P., Priddle, J., Brandon, M.A., Brierley, A.S. & Hofmann, E. 1998 Interannual variability of the South Georgia marine ecosystem: biological and physical sources of variation in the abundance of krill. *Fish. Oceanogr.* 7: 381-90.
- Mykles, D.L. & Skinner, D.M. 1985 Muscle atrophy and restoration during molting. In: Wenner, A.M. *Crustacean Issues 3: factors in adult growth*. A.A. Balkema, Rotterdam.
- Nicol, S. 1987 Some limitations on the use of the lipofuscin ageing technique. *Mar. Biol.* 93 (4): 609-614.
- Nicol, S. 1990 The age-old problem of krill longevity. *Bioscience*. 40 (11): 833-836.
- Nicol, S. 2000 Understanding krill growth and aging: the contribution of experimental studies. *Can. J. Fish. Aquat. Sci.* 57 (3): 168-177.
- Nicol, S. 2003 Living krill, zooplankton and experimental investigations. Proceedings of the International Workshop on Understanding Living Krill for Improved Management and Stock Assessment. *Marine and Freshwater Behaviour and Physiology* 36(4): 191-205.
- Nicol, S. 2006 Krill, currents and sea ice: *Euphausia superba* and its changing environment. *Bioscience*. 56 (2): 111-120.
- Nicol, S. 2008 Krill Management Pt. I. *Fishing News International*. June, 40-41.
- Nicol, S. & Endo, Y. 1999 Krill fisheries: development, management and ecosystem implications. *Aquat. Living Resour.* 12 (2): 105-120.
- Nicol, S. & Stolp, M. 1991 Moulting, feeding and fluoride concentration of the Antarctic krill, *Euphausia superba* Dana. *J. Crustacean Biol.* 11: 10-16.

- Nicol, S., Stolp, M., Cochran, T., Geijssels, P. & Marshall, J. 1992 Growth and shrinkage of Antarctic krill *Euphausia superba* from the Indian Ocean sector of the Southern Ocean during summer. *Mar. Ecol. Prog. Ser.* 89: 175-181.
- Nicol, S., Stolp, M. & Hosie, G.W. 1991 Accumulation of fluorescent age pigments in a laboratory population of Antarctic krill *Euphausia superba* Dana. *J. Exp. Mar. Biol. Ecol.* 146 (2): 153-161.
- Nicol, S., Constable, A.J. & Pauly, T. 2000 Estimates of circumpolar abundance of Antarctic krill based on recent acoustic density measurements. *CCAMLR Sci.* 7: 1-13.
- Nilsson, D.E. & Kelber, A. 2007 A functional analysis of compound eye evolution. *Arthropod Structure & Development: Origin and Evolution of Arthropod Visual Systems (Part II)* 36 (4): 373-385.
- Nishino, Y. and Kawamura, A. 1994 Winter gut contents of Antarctic krill (*Euphausia superba*) collected in the South Georgia area. *Proc. NIPR Symp. Polar Biol.* 7: 82-90.
- Nordhausen, W., Huntley, M. & Lopez, M.D.G. 1992 RACER: cannivory by *Euphausia superba* during the Antarctic winter. *Antarctic J. U.S.* 27 (5): 181-183.
- O'Donovan, V. & Tully, O. 1996 Lipofuscin (age pigment) as an index of crustacean age: correlation with age, temperature and body size in cultured juvenile *Homarus gammarus* L. *J. Exp. Mar. Biol. Ecol.* 207 (1-2): 1-14.
- Odselius R. & Elofsson R. 1981 The basement membrane of the insect and crustacean compound eye: Definition, fine structure, and comparative morphology. *Cell and Tissue Research.* 216 (1): 205-214.
- Opalinski, K.W. 1991 Respiratory metabolism and metabolic adaptations of Antarctic krill *Euphausia superba*. *Pol. Arch. Hydrobiol.* 38 (2): 183-263.
- O'Sullivan, D. 1983 Fisheries of the Southern Ocean. *Aust. Fish.* 42: 4-11.
- Pakhomov, E.A. 1995a Demographic studies of Antarctic krill (*Euphausia superba*) in the Cooperation and Cosmonaut seas (Indian sector of the Southern Ocean). *Mar. Ecol. Prog. Ser.* 119: 45-61.
- Pakhomov, E.A. 1995b Natural age-dependent mortality rates of Antarctic krill *Euphausia superba* Dana in the Indian sector of the Southern Ocean. *Polar Biol.* 15(1): 69-71.

- Pakhomov, E.A., Fuentes, V., Schloss, I., Atencio, A. & Esnal, G.B. 2003 Beaching of the tunicate *Salpa thompsoni* at high levels of suspended particulate matter in the Southern Ocean. *Polar Biol.* 26: 427-431.
- Parrish, J.K. & Hamner, W.M. (Ed) 1997 *Animal groups in three dimensions*. Cambridge University Press, Cambridge, England.
- Passmore, A. J., Jarman, S. N., Swadling, K. M., Kawaguchi, S., McMinn, A. & Nicol, S. 2006 DNA as a Dietary Biomarker in Antarctic Krill, *Euphausia superba*. *Mar. Biotech.* 8 (6), 686-696.
- Pauly, D. & David, N. 1981 ELEFAN: a BASIC program for the objective assessment of growth parameters from length frequency data. *Meeresforschung*. 28: 205-211.
- Pauly, T., Higginbottom, I., Nicol, S. & de la Mare, W. 1996 Results of a hydroacoustic survey of Antarctic krill populations in CCAMLR Division 58.4.1 carried out in January to April 1996. SC-CCAMLR WG-EMM-96/28.
- Perssinotto, R., Gurney, L. & Pakhomov, E.A. 2000 Contribution of heterotrophic material and diet to the energy budget of krill, *Euphausia superba*. *Mar. Biol.* 136 (1): 129-135.
- Pieters, H., Kluytmans, J.H., Zandee, D.I., & Cadée, G.C. 1980 Tissue composition and reproduction in *Mytilus edulis* in relation to food availability. *Netherlands J. Sea Res.* 14 (3-4): 349-361.
- Poleck, T.P. & Denys, C.J. 1982 Effect of temperature on the molting, growth and maturation of the Antarctic krill *Euphausia superba* (Crustacea: Euphausiacea) under laboratory conditions. *Mar. Biol.* 70: 255-265.
- Porta, E.A. 2002 Pigments in Aging: an Overview. *Annals New York Academy Sciences*. 959: 57-65.
- Quetin, L.B., & Ross, R.M. 1991 Behavioural and physiological characteristics of Antarctic krill *Euphausia superba* Dana. *Am. Zool.* 31: 49-63.
- Quetin L.B. & Ross, R.M 2001 Environmental variability and its impact on the reproductive cycle of Antarctic krill. *Am. Zool.* 41: 74-89.
- Quetin, L.B. & Ross, R.M. 2003 Episodic recruitment in Antarctic krill *Euphausia superba* in the Palmer LTER study region. *Mar. Ecol. Prog. Ser.* 259: 185-200.
- Quetin, L.B., Ross, R.M. & Clarke, A. 1994 Krill Energetics: seasonal and environmental aspects of the physiology of *Euphausia superba*. In: El-Sayed S.Z. (Ed.) *Southern Ocean ecology: the BIOMASS perspective*. Cambridge University Press, Cambridge. pp 165-184.

- Quetin, L.B., Ross, R.M., Frazer, T.K., Amsler, M.O., Wyatt Evens, C. & Oakes, S.A. 2003 Growth of larval krill, *Euphausia superba*, in fall and winter West of the Antarctic Peninsula. *Mar. Biol.* 143 (5): 833-843.
- R Development Core Team 2008 R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL <http://www.R-project.org>.
- Radu, R.A., Yuan, Q., Hu, J., Peng, J.H., Lloyd, M., Nusinowitz, S., Bok, D. & Travis, G.H. 2008 Accelerated Accumulation of Lipofuscin Pigments in the RPE of a Mouse Model for ABCA4-Mediated Retinal Dystrophies following Vitamin A Supplementation. *Inv. Ophthalmol. Vis. Sci.* 49: 3821-3829.
- Rakusa-Suszczewski, S. 1990 Seasonal changes in respiration and biomass of *Euphausia superba* Dana from Admiralty Bay (South Shetland Islands). *Pol. Arch. Hydrobiol.* 37: 305-311.
- Rakusa-Suszczewski, S. 1981 Polish Antarctic Expedition 1975/76. In: El-Sayed, S.Z. (Ed.) Biological Investigations of Marine Antarctic Systems and Stocks (BIOMASS) Vol. II. SCAR & SCOR. Scott Polar Research Institute, Cambridge, England. pp. 151-155.
- Rakusa-Suszczewski, S. & Opalinski, K.W. 1978 Oxygen consumption in *Euphausia superba*. *Pol. Arch. Hydrobiol.* 25: 633-641.
- Reid, K. 2001 Growth of Antarctic krill *Euphausia superba* at South Georgia. *Mar. Biol.* 138 (1): 57-62.
- Reid, K., Measures, J. 1998 Determining the sex of Antarctic krill *Euphausia superba* using carapace measurements. *Polar Biol.* 19(2):145-147.
- Reid, K., Jessopp, M. J., Barretta, M. S., Kawaguchi S., Siegel V. & Goebeld M. E. 2004 Widening the net: spatio-temporal variability in the krill population structure across the Scotia Sea. *Deep Sea Research Part II: Topical Studies in Oceanography* 51 (12-13) pp: 1275-1287.
- Ritz, D.A. 1994 Social aggregation in pelagic invertebrates. *Adv. Mar. Biol.* 30: 156-216.
- Ritz, D.A. 2002 Comment on Alonzo and Mangel (2001) Survival strategies and growth of krill: avoiding predators in space and time. *Mar. Ecol. Prog. Ser.* 244: 307-308.
- Ritz, D.A., Cromer, L., Swadling, K.M., Nicol, S. & Osborn J. 2002 Heart rate as a measure of stress in Antarctic krill, *Euphausia superba*. *J. Mar. Biol. Assoc. U.K.* 82: 183-184.

- Robson, S.K.A. Vickers M., Blows M.W. & Crozier R.H. 2006 Age determination in individual wild-caught *Drosophila serrata* using pteridine concentration. *J. Exp. Biol.* 209: 3155-3163.
- Roer, R. & Dillaman, R. 1984 The structure and calcification of the crustacean cuticle. *Am. Zool.* 24: 893-909.
- Roff, J.C., Kroetsch, J.T. & Clarke, A.J. 1994 A radiochemical method for secondary production in planktonic Crustacea based on chitin synthesis. *J. Plankton. Res.* 16: 961-976.
- Rosenberg, A.A., Beddington, J.R. & Basson, M. 1986 Growth and longevity of krill during the first decade of pelagic whaling. *Nature.* 324: 152-154.
- Rosenberry, R. 1996. ALGAMAC-2000. *Shrimp News International* 21(1): 4.
- Ross, R.M. & Quetin, L.B. 1988 *Euphausia superba*: a critical review of estimates of annual production. *Comp. Biochem. Physiol.*, 90B (3): 499-505.
- Ross, R.M. & Quetin, L.B. 2000 Reproduction in Euphausiacea. In: Everson, I. (Ed.) *Krill: biology, ecology and fisheries*. Blackwell Science, Cambridge, pp: 150-181.
- Ross, R.M., Quetin, L.B., Baker, K.S., Vernet, M. & Smith, R.C. 2000 Growth limitation in young *Euphausia superba* under field conditions. *Limnol. Oceanogr.* 45: 31-43.
- Saborowski R., Salomon, M., Buchholz, F. The physiological response of Northern krill 2000 (*Meganyctiphanes norvegica*) to temperature gradients in the Kattegat. *Hydrobiol.* 426 (1-3): 157-160.
- Secor D.H., Dean J.M., & Campana S.E. (1995) Recent developments in fish otolith research. University of South Carolina Press, Columbia, SC.
- Seibel, B.A. & Drazen J.C. The rate of metabolism in marine animals: environmental constraints, ecological demands and energetic opportunities. *Phil. Tran. Royal Soc. B: Biol. Sci.* 362 (1487): 2061-2078.
- Segawa, S., Kato, M. & Murano, M. 1982 Respiration and ammonium excretion of Antarctic krill, *Euphausia superba* Dana. *Trans. Tokyo Univ. Fish.* 5: 177-187.
- Sheehy, M.R.J. 1990a Potential of morphological lipofuscin age-pigment as an index of crustacean age. *Mar. Biol.* 107 (3): 439-442.
- Sheehy, M.R.J. 1990b Widespread occurrence of fluorescent morphological lipofuscin in the crustacean brain. *J. Crust. Biol.* 10: 613-622.

- Sheehy, M.R.J. 1990c Individual variation in, and the effect of rearing temperature and body size on, the concentration of fluorescent morphological lipofuscin in the brains of freshwater crayfish, *Cherax cuspidatus* (Crustacea: Parastacidae). *Comp. Biochem. Physiol. A: Physiol.* 96 (2): 281-286.
- Sheehy, M.R., J. 1992 Lipofuscin age-pigment accumulation in the brains of ageing field- and laboratory-reared crayfish *Cherax quadricarinatus* (von Martens) (Decapoda : Parastacidae). *J. Exp. Mar. Biol. Ecol.* 161 (1): 79-89.
- Sheehy, M.R.J. 1996 Quantitative comparison of in situ lipofuscin concentration with soluble autofluorescence intensity in the crustacean brain. *Exp. Geront.* 31 (3): 421-432.
- Sheehy, M.R.J. 2008 Questioning the use of biochemical extraction to measure lipofuscin for age determination of crabs: Comment on Ju et al. (1999, 2001). *Mar. Ecol. Prog. Ser.* 353: 303-306.
- Sheehy, M.R.J. & Ettershank, G. 1988 Extractable age pigment-like autofluorescence and its relationship to growth and age in the water-flea *Daphnia carinata* King (Crustacea, Cladocera). *Aust. J. Zool.* 36 (6): 611-625.
- Sheehy, M.R.J. & Roberts, B.E. 1991 An alternative explanation for anomalies in "soluble lipofuscin" fluorescence data from insects, crustaceans, and other aquatic species. *Exp. Geront.* 26(5): 495-509.
- Sheehy, M.R.J., Greenwood, J.G. & Fielder, D.R. 1995 Lipofuscin as a record of "rate of living" in an aquatic poikilotherm. *J. Gerontol. Ser. A: Biol. Sci. & Med. Sci.* 50 (6): 327-336.
- Sheehy, M., Caputi, N., Chubb, C. & Belchier, M. 1998 Use of lipofuscin for resolving cohorts of western rock lobster (*Panulirus cygnus*). *Can. J. Fish. Aquat. Sci.* 55 (4): 925-936.
- Sheen, S.S. & Wu, S.W. 2002 Essential Fatty Acid Requirements of Juvenile Mud Crab, *Scylla serrata* (Forsk., 1775) (Decapoda, Scyllaridae). *Crustaceana*, 75 (11): 1387-1401.
- Shin, H.-C. & Nicol, S. 2002 Using the relationship between eye diameter and body length to detect the effects of long term starvation on juvenile Antarctic krill *Euphausia superba*. *Mar. Ecol. Prog. Ser.* 239: 157-167.
- Shin, H.-C., Nicol, S. & King, R.A 2003 Nucleic acid content as a potential growth rate estimator of Antarctic krill; results from field-caught krill from the Indian sector of the Southern Ocean. *Mar. Freshwater Behav. Physiol. Spec. Iss. Understanding living krill for improved management and stock assessment.* 36 (4): 295-305.



- Siegel, V. 1987 Age and growth of Antarctic Euphausiacea (Crustacea) under natural conditions. *Mar. Biol.* 96: 483-495.
- Siegel, V. 2000 Krill (Euphausiacea) life history and aspects of population dynamics. *Can. J. Fish. Aquat. Sci.* 57: 130-150.
- Siegel, V. 2005 Distribution and population dynamics of *Euphausia superba*: summary of recent findings. *Polar Biol.* 29: 1-22.
- Siegel, V. & Nicol, S. 2000 Population parameters. In: Everson, I. (Ed.) *Krill biology, ecology and fisheries*. Blackwell Science Ltd, Oxford, U.K. pp. 102-149.
- Skinner, D.M. 1966 Breakdown and reformation of somatic muscle during the molt cycle of the land crab, *Geocarcinus lateralis*. *J. Exp. Zool.* 163: 115-124.
- Smetacek, V. & Nicol, S. 2005 Polar ocean ecosystems in a changing world. *Nature* 437: 362-368.
- Smith, M.T. & Addison, J.T. 2003 Methods for stock assessment of crustacean fisheries. *Fish. Res.* 65, (1-3): 231-256.
- Smith, W.O. & Nelson, D.M. 1990 Phytoplankton growth and new production in the Weddell Sea marginal ice zone during austral spring and autumn. *Limnol. Oceanogr.* 35: 809-821.
- Smith, W.O. & Sakshaug, E. 1990 Polar phytoplankton. In: Smith, W.O. (Ed.) *Polar oceanography*. Academic Press, New York, pp: 477-526.
- Stankovic A., Borsuk, P., Koper, M. & Weglenski P. 2002 Studies on *Ephelota* sp., an epizoic suctorian found on Antarctic krill. *Polar Biol.* 25 (11): 827-832.
- Storey, K.B. 1996 Oxidative stress: animal adaptations in nature. *Braz. J. Med. Biol. Res.* 29: 1715-1733.
- Sun, S. 1987 Using Antarctic krill as an indicator of environmental interannual change. *Korean J. Polar Res.* 8 (1, 2): 97-103.
- Sun S. & Wang, R. 1996 Study on the relationship between the diameter of the compound eye and the growth of Antarctic krill. *Antarct. Res.* 7: 87-93.
- Sun, S., de la Mare, W., & Nicol, S. 1995 The compound eye as an indicator of age and shrinkage in Antarctic krill. *Antarct. Sci.* 7: 387-392.
- Swadling, K.M., Ritz, D.A., Nicol, S. Osborn, J.E. & Gurney, L.J. 2005 Respiration rate and cost of swimming for Antarctic krill, *Euphausia superba*, in large groups in the laboratory. *Mar. Biol.* 146: 1169-1175.

- Takahashi, K.T., Kawaguchi, S., Kobayashi, M. & Toda, T. 2003 Parasitic eugregarines change their spatial distribution within the host digestive tract of Antarctic krill, *Euphausia superba*. *Polar Biol.* 26 (7): 468-473.
- Takahashi, K.T., Kawaguchi, S., Kobayashi, M. & Toda, T. 2004 The variability in abundance of eugregarines living in the Antarctic krill. *Polar Biosci.* 17: 16-25.
- Tarling, G. A., Cuzin-Roudy, J. & Buchholz, F. 1999 Vertical migratory behaviour in the northern krill (*Meganyctiphanes norvegica*) is influenced by moult and reproductive processes. *Mar. Ecol. Prog. Ser.* 190: 253-262.
- Tarling, G.A., & Johnson, M.L. 2006 Satiation gives krill that sinking feeling. *Current Biol.* 16 (3): R83-R84.
- Tarling, G.A., Shreeve, R.S., Hirst, A.G., Atkinson, A., Pond, D.W., Murphy, E.J., & Watkins, J.L. 2006 Natural growth rates in Antarctic krill (*Euphausia superba*): I. Improving methodology and predicting intermolt period. *Limnol. Oceanogr.* 51(2): 959-972.
- Terman, A., Brunk, U.T. 1998 Ceroid/lipofuscin formation in cultured human fibroblasts: the role of oxidative stress and lysosomal proteolysis. *Mech. Ageing Dev.* 104, pp.277-291.
- Teschke, M., Kawaguchi, S. & Meyer, B. 2007 Simulated light regimes affect feeding and metabolism of Antarctic krill, *Euphausia superba*. *Limnol. Oceanogr.*, 52(3): 1046-1054.
- Teschke, M. Kawaguchi, S. & Meyer, B. 2008 Effects of simulated light regimes on maturity and body composition of Antarctic krill, *Euphausia superba*. *Mar. Biol.* 154 (2) 315-324.
- Thomas, P. & Ikeda, T. 1987 Sexual regression, shrinkage, re-maturation and growth of spent female *Euphausia superba* in the laboratory. *Mar. Biol.* 95: 357-363.
- Torres, J.J., Aarset, A.V., Donnelly, J., Hopkins, T.L. Lancraft, T.M. & Ainley, D.G. 1994 Metabolism of Antarctic micronektonic Crustacea as a function of depth of occurrence and season. *Mar. Ecol. Prog. Ser.* 113: 207-219.
- Trathan, P.N., Brierley, A.S., Brandon, M.A., Bone, D.G. Goss, C. Grant, S.A., Murphy, E.J. & Watkins, J.L. 2003 Oceanographic variability and changes in Antarctic krill (*Euphausia superba*) abundance at South Georgia. *Fisheries Oceanography.* 12 (6): 569-583.
- Tully, O., O'Donovan, V. & Fletcher, D. 2000 Metabolic rate and lipofuscin accumulation in juvenile European lobster (*Homarus gammarus*) in

relation to simulated seasonal changes in temperature. *Mar. Biol.* 137 (5-6): 1031-1040.

Udenfriend, S. 1962 *Fluorescence assay in biology and medicine, Vol I.* Academic Press, New York.

Udenfriend S 1969 *Fluorescence assay in biology and medicine, Vol II.* Academic Press, New York.

Vernet, M., Hunter J. R. & Vetter, R. D. 1988 Accumulation of age pigments (lipofuscin) in two cold-water fishes. *Fish. Bull.* 86 (2): 401-407.

Vila, Y., Medina, A., Megina, C., Ramos, F. & Sobrino, I. 2000 Quantification of the age-pigment lipofuscin in brains of known-age, pond-reared prawns *Panaeus japonicus* (Crustacea, Decapoda). *J. Exp. Zool.* 286 (2):120-130.

Virtue, P., Nichols, P.D., Nicol, S., & Hosie, G. 1996 Reproductive trade-off in male Antarctic krill *Euphausia superba*. *Mar. Biol.* 126 (3): 521-527.

Virtue, P., Nicholls, P. D. & Nicol, S. 1997 Dietary related mechanisms of survival in *Euphausia superba*: changes during long term starvation and bacteria as a possible source of nutrition. pp 193-201. In: *Antarctic Communities - species, structure and survival*. Battaglia, B., Valencia, J. and Walton, D. W. H. (eds.). Cambridge University Press. 464 pp.

Virtue P., Kawaguchi S., McIvor J., Nicol S., Wotherspoon S., Brown M., Casper R., Davenport S., Finlay L., Foster J., Yoshida T., Yoshiki T. 2009 Krill growth and condition in Western Indian Ocean sector of the Southern Ocean 30-80 oE in austral summer 2006. *Deep Sea Res.* (in press).

Voet, D. & Voet, J.G. 2004 *Biochemistry* (3rd ed.). John Wiley & Sons.

Wahle, R.A., Tully, O. & O'Donovan, V. 1996(a) Age determination in crustaceans: fluorescing age pigments as a potential tool in population studies. 24. Annual Benthic Ecology Meeting, Columbia, SC (USA), 7-10 Mar 1996.

Wahle, R.A., Tully, O. & O'Donovan, V. 1996(b) Lipofuscin as an indicator of age in crustaceans: analysis of the pigment in the lobster, *H. americanus*. *Mar. Ecol. Prog. Ser.* 138: 117-123.

Watkins, J.L., Morris, D.J. & Ricketts, C. 1985 Nocturnal changes in the mean length of a euphausiid population: vertical migration, net avoidance, or experimental error? *Mar. Biol.* 86 (2): 123-127.

- Weisberg, S. 2003 Using hard-part increment data to estimate age and environmental effects. *Can. J. Fish. Aquat. Sci.* 50 (6): 1229-1237.
- Wenner, A.M. 1985 Ed. *Crustacean issues 3: factors in adult growth*. A.A. Balkema Press, Rotterdam, The Netherlands.
- Wing G.L., Blanchard, G.C. & Weiter, J.J. The topography and age relationship of lipofuscin concentration in the retinal pigment epithelium. *Inv. Opth. & Vis. Sci.* 17: 601-607.
- Withers, P.C. 1992 *Comparative animal physiology*. Saunders College Publishing, New York, USA. 949 pp.
- Yamaoka, L.H. & Skinner, D.M. 1974 Synthesis of structural proteins in regenerating and non-regenerating crustacean muscle in the molt cycle. *J. Cell. Biol.* 63: 381a.
- Yamamota, Y., Honda, K. & Tatsukawa, R. 1987 Heavy metal accumulation in Antarctic krill *Euphausia superba*. *Proc. NIPR Symp. Polar Biol.* 1: 198-204.
- Yin, D.Z. 1996 Biochemical basis of lipofuscin, ceroid and age pigment-like fluorophores. *Free Rad. Biol. Med.* 21 (6): 871-888.
- Zielinski, S. & Pörtner, H-O. 2000 Oxidative stress and antioxidative defense in cephalopods: a function of metabolic rate or age? *Comp. Biochem. Physiol. B.* 125: 147-160.



## Appendix I: Fit of von Bertalanffy growth models

---

*Code in R (output follows)*

```
# Angela's pigment analyses
# part 1 Cumulative pigment vs age
# fit of VB models
# multivariate analysis using asreml

library(lattice)

data.df <- read.csv(file="Part1.csv")

summary(data.df)

data.df$pigment.1[data.df$Age_mth==1] <-
data.df$pigment.1[data.df$Age_mth==1]/c(12,13)
data.df$pigment.2[data.df$Age_mth==1] <-
data.df$pigment.2[data.df$Age_mth==1]/c(12,13)
data.df$pigment.3[data.df$Age_mth==1] <-
data.df$pigment.3[data.df$Age_mth==1]/c(12,13)
data.df$wetwteyes[data.df$Age_mth==1] <-
data.df$wetwteyes[data.df$Age_mth==1]/c(12,13)

# plot graphs

xyplot(pigment.1 ~ Age_mth | sex, data=data.df, type="p")

xyplot(pigment.2 ~ Age_mth | sex, data=data.df, type="p")

xyplot(pigment.3 ~ Age_mth | sex, data=data.df, type="p")

xyplot(CL ~ Age_mth | sex, data=data.df, type="p")

xyplot(wetwteyes ~ Age_mth | sex, data=data.df, type="p")

# calculate means by age class (anova's at end suggest no significant effect of sex
for pigment.3 and wetwteyes)

data.df$age.f <- as.factor(data.df$Age_mth)

age.fs <- factor(rep(as.vector(levels(data.df$age.f)),times=2),
levels=levels(data.df$age.f))
sex.fs <- factor(rep(levels(data.df$sex),each=N.age), levels=levels(data.df$sex))

mean.wetwteyes <-
as.vector(tapply(data.df$wetwteyes,INDEX=list(data.df$age.f), FUN=mean))
SD.wetwteyes <- (as.vector(tapply(data.df$wetwteyes,INDEX=list(data.df$age.f),
FUN=var)))^0.5
```

```

N.wetwteyes <-
as.vector(tapply(rep(1,length(data.df$wetwteyes)),INDEX=list(data.df$age.f),
FUN=sum))

mean.pig3 <- as.vector(tapply(data.df$pigment.3,INDEX=list(data.df$age.f),
FUN=mean))
SD.pig3 <- (as.vector(tapply(data.df$pigment.3,INDEX=list(data.df$age.f),
FUN=var)))^0.5
N.pig3 <-
as.vector(tapply(rep(1,length(data.df$pigment.3)),INDEX=list(data.df$age.f),
FUN=sum))

CLu.pig3 <- mean.pig3+2*SD.pig3/(N.pig3^0.5)
CLl.pig3 <- mean.pig3-2*SD.pig3/(N.pig3^0.5)

CLu.wetwteyes <- mean.wetwteyes+2*SD.wetwteyes/(N.wetwteyes^0.5)
CLl.wetwteyes <- mean.wetwteyes-2*SD.wetwteyes/(N.wetwteyes^0.5)

# model growth in pigments, wetwteyes and CL using a von Bertalanffy after log
transformation

"VB.pred" <- Pred.v <- function(par,xval) {
  cbind(xval,par[1]*(1-exp(-par[2]*(xval/12-par[3]))))}

age.v <- c(1,12,36,48)

data.vb.df <- data.df

# Growth for pigment.3

data.df$yv <- data.df$pigment.3
vpar <- rep(0,3)
vpar[1] <- 1.1*max(data.df$yv)
vpar.m <- 0.9*min(data.df$yv)
data.vb.df$y <- log(1-data.df$yv/vpar[1])

lm.01 <-lm(formula= y ~ Age_mth, data=data.vb.df)
vpar[2] <- -12*lm.01$coef[2]

VB.fit <- nls(formula= yv ~ Linf*(1-exp(-k*(Age_mth/12-t0))),
  data=data.df, start=list(Linf=vpar[1],k=vpar[2],t0=0.0))

summary(VB.fit)

par <- coef(VB.fit)

# plot VB fit

Pred.v <- VB.pred(par=par,xval=seq(1:50))

```

Pred.v

```
plot(y=Pred.v[,2], x=Pred.v[,1], xlab="Age (months)", ylab="pigment.3",
type="l", ylim=c(vpar.m,vpar[1]),
lwd=2, col="grey")
points(y=data.df$yv, x=data.df$Age_mth, pch=1)
points(y=mean.pig3, x=age.v, pch=2, cex=1.5, col="grey")
for (i in (1:4)) {
segments(y1=CLu.pig3[i], x1=age.v[i], y0=CLl.pig3[i], x0=age.v[i], col="grey")}
title(main="VB curve, values, means, +/- 2SE(mean)")
```

```
par.pig3 <- par
#SD.pig3 <-
(sum(residuals(VB.fit)[data.df$Age_mth!=1]^2)/sum(as.integer(data.df$Age_mth!=1)))^0.5
SD.pig3 <- (deviance(VB.fit)/df.residual(VB.fit))^0.5
```

# Growth for wetwteyes

```
data.df$yv <- data.df$wetwteyes
vpar <- rep(0,3)
vpar[1] <- 1.1*max(data.df$yv)
vpar.m <- 0.9*min(data.df$yv)
data.vb.df$y <- log(1-data.df$yv/vpar[1])

lm.01 <-lm(formula= y ~ Age_mth, data=data.vb.df)
vpar[2] <- -12*lm.01$coef[2]

VB.fit <- nls(formula= yv ~ Linf*(1-exp(-k*(Age_mth/12-t0))),
data=data.df, start=list(Linf=vpar[1],k=vpar[2],t0=0.0))

summary(VB.fit)

par <- coef(VB.fit)

# plot VB fit
```

```
Pred.v <- VB.pred(par=par,xval=seq(1:50))
Pred.v
```

```
plot(y=Pred.v[,2], x=Pred.v[,1], xlab="Age (months)", ylab="wetwteyes",
type="l", ylim=c(vpar.m,vpar[1]),
lwd=2, col="grey")
points(y=data.df$yv, x=data.df$Age_mth, pch=1)
points(y=mean.wetwteyes, x=age.v, pch=2, cex=1.5, col="grey")
for (i in (1:4)) {
segments(y1=CLu.wetwteyes[i], x1=age.v[i], y0=CLl.wetwteyes[i], x0=age.v[i],
col="grey")}
title(main="VB curve, values, means, +/- 2SE(mean)")
```



```

par.wetwteyes <- par
#SD.wetwteyes <-
(sum(residuals(VB.fit)[data.df$Age_mth!=1]^2)/sum(as.integer(data.df$Age_mth!
=1)))^0.5
SD.wetwteyes <- (deviance(VB.fit)/df.residual(VB.fit))^0.5

# calculate means by age and sex class (anova's at end suggest significant effect of
sex for pigment.1 and CL)
levels(data.df$sex)
age.fs <- factor(rep(as.vector(levels(data.df$age.f)),times=3),
levels=levels(data.df$age.f))
sex.fs <- factor(rep(levels(data.df$sex),each=N.age), levels=levels(data.df$sex))

mean.CL <- as.vector(tapply(data.df$CL,INDEX=list(data.df$age.f,data.df$sex),
FUN=mean))
SD.CL <- (as.vector(tapply(data.df$CL,INDEX=list(data.df$age.f,data.df$sex),
FUN=var)))^0.5
N.CL <-
as.vector(tapply(rep(1,length(data.df$CL)),INDEX=list(data.df$age.f,data.df$sex),
FUN=sum))

mean.pig1 <-
as.vector(tapply(data.df$pigment.1,INDEX=list(data.df$age.f,data.df$sex),
FUN=mean))
SD.pig1 <-
(as.vector(tapply(data.df$pigment.1,INDEX=list(data.df$age.f,data.df$sex),
FUN=var)))^0.5
N.pig1 <-
as.vector(tapply(rep(1,length(data.df$pigment.1)),INDEX=list(data.df$age.f,data.d
f$sex), FUN=sum))
N.age <- length(levels(data.df$age.f))
mean.pig2 <-
as.vector(tapply(data.df$pigment.2,INDEX=list(data.df$age.f,data.df$sex),
FUN=mean))
SD.pig2 <-
(as.vector(tapply(data.df$pigment.2,INDEX=list(data.df$age.f,data.df$sex),
FUN=var)))^0.5
N.pig2 <-
as.vector(tapply(rep(1,length(data.df$pigment.2)),INDEX=list(data.df$age.f,data.d
f$sex), FUN=sum))

CLu.pig1 <- mean.pig1+SD.pig1/(N.pig1^0.5)
CLl.pig1 <- mean.pig1-SD.pig1/(N.pig1^0.5)
CLu.pig2 <- mean.pig2+SD.pig2/(N.pig2^0.5)
CLl.pig2 <- mean.pig2-SD.pig2/(N.pig2^0.5)
CLu.CL <- mean.CL+SD.CL/(N.CL^0.5)
CLl.CL <- mean.CL-SD.CL/(N.CL^0.5)

```

```

# Growth for pigment.1 & sex!=f
vpar <- rep(0,3)
vpar[1] <- 1.1*max(data.df$pigment.1)
vpar.m <- 0.9*min(data.df$pigment.1)

data.m.df <- data.df[data.df$sex!="f",]

data.m.df$yv <- data.m.df$pigment.1
data.vb.df$y <- log(1-data.m.df$yv/vpar[1])

lm.01 <- lm(formula= y ~ Age_mth, data=data.vb.df)
vpar[2] <- -12*lm.01$coef[2]

VB.fit <- nls(formula= yv ~ Linf*(1-exp(-k*(Age_mth/12-t0))),
  data=data.m.df, start=list(Linf=vpar[1],k=vpar[2],t0=0.0))

summary(VB.fit)

par <- coef(VB.fit)

# plot VB fit

Pred.v.m <- VB.pred(par=par,xval=seq(1:50))

# Growth for pigment.1 & sex!=m

data.f.df <- data.df[data.df$sex!="m",]

data.f.df$yv <- data.f.df$pigment.1
data.vb.df$y <- log(1-data.f.df$yv/vpar[1])

lm.01 <- lm(formula= y ~ Age_mth, data=data.vb.df)
vpar[2] <- -12*lm.01$coef[2]

VB.fit <- nls(formula= yv ~ Linf*(1-exp(-k*(Age_mth/12-t0))),
  data=data.f.df, start=list(Linf=vpar[1],k=vpar[2],t0=0.0))

summary(VB.fit)

par <- coef(VB.fit)

# plot VB fit

Pred.v.f <- VB.pred(par=par,xval=seq(1:50))

plot(y=Pred.v.m[,2], x=Pred.v.m[,1], xlab="Age (months)", ylab="pigment.1",
type="l", ylim=c(vpar.m,vpar[1]),

```

```

lwd=2, col="grey")
lines(y=Pred.v.f[,2], x=Pred.v.f[,1], lty=2, col="green", lwd=2)
points(y=data.m.df$yv, x=data.m.df$Age_mth, pch=1, col="grey")
points(y=data.f.df$yv, x=data.f.df$Age_mth, pch=4, col="green")
points(y=mean.pigl[10:12], x=age.v[2:4], pch=2, cex=1.5, col="grey")
points(y=mean.pigl[2:4], x=age.v[2:4], pch=2, cex=1.5, col="green")
for (i in (10:12)) {
  segments(y1=CLu.pigl[i], x1=age.v[i-8], y0=CLl.pigl[i], x0=age.v[i-8],
  col="grey")}
for (i in (2:4)) {
  segments(y1=CLu.pigl[i], x1=age.v[i], y0=CLl.pigl[i], x0=age.v[i],
  col="green")}}

legend(x=0, y=0.8*vpar[1], legend=c("male", "female"), lty=c(1,2),
col=c("grey", "green"), pch=c(1,4))
title(main="VB curve, values, means, +/- 2SE(mean)")

# Growth for CL & sex!=f
vpar <- rep(0,3)
vpar[1] <- 1.1*max(data.df$CL)
vpar.m <- 0.9*min(data.df$CL)

data.m.df <- data.df[data.df$sex!="f",]

data.m.df$yv <- data.m.df$CL
data.vb.df$y <- log(1-data.m.df$yv/vpar[1])

lm.01 <- lm(formula= y ~ Age_mth, data=data.vb.df)
vpar[2] <- -12*lm.01$coef[2]

VB.fit <- nls(formula= yv ~ Linf*(1-exp(-k*(Age_mth/12-t0))),
  data=data.m.df, start=list(Linf=vpar[1],k=vpar[2],t0=0.0))

summary(VB.fit)

par.CL.f <- coef(VB.fit)
SD.CL.f <- (deviance(VB.fit)/df.residual(VB.fit))^0.5

# plot VB fit

Pred.v.m <- VB.pred(par=par.CL.f,xval=seq(1:50))

# Growth for CL & sex!=m

data.f.df <- data.df[data.df$sex!="m",]

data.f.df$yv <- data.f.df$CL

```

```

data.vb.df$y <- log(1-data.f.df$yv/vpar[1])

lm.01 <-lm(formula= y ~ Age_mth, data=data.vb.df)
vpar[2] <- -12*lm.01$coef[2]

VB.fit <- nls(formula= yv ~ Linf*(1-exp(-k*(Age_mth/12-t0))),
  data=data.f.df, start=list(Linf=vpar[1],k=vpar[2],t0=0.0))

summary(VB.fit)

par.CL.m <- coef(VB.fit)
SD.CL.m <- (deviance(VB.fit)/df.residual(VB.fit))^0.5

# plot VB fit

Pred.v.f <- VB.pred(par=par.CL.m,xval=seq(1:50))

plot(y=Pred.v.m[,2], x=Pred.v.m[,1], xlab="Age (months)", ylab="CL", type="l",
  ylim=c(vpar.m,vpar[1]),
  lwd=2, col="grey")
lines(y=Pred.v.f[,2], x=Pred.v.f[,1], lty=2, col="green", lwd=2)
points(y=data.m.df$yv, x=data.m.df$Age_mth, pch=1, col="grey")
points(y=data.f.df$yv, x=data.f.df$Age_mth, pch=4, col="green")
points(y=mean.CL[10:12], x=age.v[2:4], pch=2, cex=1.5, col="grey")
points(y=mean.CL[2:4], x=age.v[2:4], pch=2, cex=1.5, col="green")
for (i in (10:12)) {
  segments(y1=CLu.CL[i], x1=age.v[i-8], y0=CLl.CL[i], x0=age.v[i-8],
  col="grey")}
for (i in (2:4)) {
  segments(y1=CLu.CL[i], x1=age.v[i], y0=CLl.CL[i], x0=age.v[i], col="green")}}

legend(x=0, y=0.8*vpar[1], legend=c("male","female"), lty=c(1,2),
  col=c("grey","green"), pch=c(1,4))
title(main="VB curve, values, means, +/- 2SE(mean)")

data.mf.df <- data.df[data.df$sex!="j",]

data.mf.df$sex <- as.factor(as.character(data.mf.df$sex))

data.mf.df$age.f <- as.factor(data.mf.df$Age_mth)

summary(data.mf.df)

# fit linear model

lm.01 <- lm(formula=pigment.1 ~ age.f+sex+age.f:sex, data=data.mf.df)

```

```
anova(lm.01)
summary(lm.01)
```

```
# fit linear model
```

```
lm.01 <- lm(formula=pigment.3 ~ age.f+sex+age.f:sex, data=data.mf.df)
```

```
anova(lm.01)
summary(lm.01)
```

```
lm.01 <- lm(formula=wetwteyes ~ age.f+sex+age.f:sex, data=data.mf.df)
```

```
anova(lm.01)
summary(lm.01)
```

```
lm.01 <- lm(formula=CL ~ age.f+sex+age.f:sex, data=data.mf.df)
```

```
anova(lm.01)
summary(lm.01)
```

```
library(asreml)
```

```
# run asreml to model correlations
```

```
asreml.mv <- asreml(fixed = cbind(pigment.3, wetwteyes) ~ trait + trait:age.f,
  rcov= ~ units:diag(trait), data=data.mf.df, na.method.Y = "exclude",
  na.method.X = "exclude", maxiter=30)
```

```
anova(asreml.mv)
(summary(asreml.mv))$varcomp
(summary(asreml.mv))$coef.fixed
```

```
asreml.mv <- asreml(fixed = cbind(pigment.3, wetwteyes) ~ trait + age.f + sex +
  trait:age.f +
  trait:sex + trait:age.f:sex,
  rcov= ~ units:diag(trait), data=data.mf.df, na.method.Y = "exclude",
  na.method.X = "exclude", maxiter=30)
```

```
anova(asreml.mv)
(summary(asreml.mv))$varcomp
(summary(asreml.mv))$coef.fixed
```

```
asreml.mv <- asreml(fixed = cbind(pigment.1, pigment.3, wetwteyes, CL) ~ trait +
  age.f + sex + trait:age.f +
  trait:sex + trait:age.f:sex,
  rcov= ~ units:diag(trait), data=data.mf.df, na.method.Y = "exclude",
```

```
na.method.X = "exclude", maxiter=30)
```

```
anova(asreml.mv)
(summary(asreml.mv))$varcomp
(summary(asreml.mv))$coef.fixed
```

```
asreml.mv <- asreml(fixed = cbind(pigment.1, pigment.3, wetwteyes, CL) ~ trait +
age.f + sex + trait:age.f +
  trait:sex + trait:age.f:sex,
  rcov = ~ units:us(trait), data = data.mf.df, na.method.Y = "exclude",
  na.method.X = "exclude", maxiter=30)
```

```
anova(asreml.mv)
(summary(asreml.mv))$varcomp
(summary(asreml.mv))$coef.fixed
```

```
asreml.mv <- asreml(fixed = cbind(pigment.3, wetwteyes) ~ trait + trait:age.f,
  rcov = ~ units:us(trait), data = data.mf.df, na.method.Y = "exclude",
  na.method.X = "exclude", maxiter=30)
```

```
asreml.mv <- asreml(fixed = cbind(pigment.3, CL, wetwteyes) ~ trait + age.f +
  trait:age.f,
  rcov = ~ units:us(trait), data = data.mf.df, na.method.Y = "exclude",
  na.method.X = "exclude", maxiter=30)
```

```
anova(asreml.mv)
(summary(asreml.mv))$varcomp
(summary(asreml.mv))$coef.fixed
```



## Appendix II: Simulation study

---

### *Code in R*

```
# Angela's pigment analyses
# part 1 Cumulative pigment vs age
# fit of VB models to simulated age 1,2,3, and 4 data
# assume Part1_mv.r has been run

"VB.pred" <- Pred.v <- function(par,xval) {
  cbind(xval,par[1]*(1-exp(-par[2]*(xval/12-par[3]))))}

age.v <- c(12,24,36,48)

# simulate Nsim krill at each age 1 to 4 yrs for
# joint distribution of wetwteyes and pigment3 assuming zero covariance

par.pig3
SD.pig3

par.wetwteyes
SD.wetwteyes

Nsim <- 1000

No.ages <- length(age.v)

VBeyes <- VB.pred(par=par.wetwteyes, xval= age.v)

VBpig3 <- VB.pred(par=par.pig3, xval= age.v)

VBCL.f <- VB.pred(par=par.CL.f, xval= age.v)

VBCL.m <- VB.pred(par=par.CL.m, xval= age.v)

wetwteyes.m <- matrix(data=rep(0,Nsim*No.ages), nrow=Nsim, ncol=No.ages)

pig3.m <- matrix(data=rep(0,Nsim*No.ages), nrow=Nsim, ncol=No.ages)

CL.f.m <- matrix(data=rep(0,Nsim*No.ages), nrow=Nsim, ncol=No.ages)

CL.m.m <- matrix(data=rep(0,Nsim*No.ages), nrow=Nsim, ncol=No.ages)

# try very small SD for pigment3

SD.pig3.save <- SD.pig3
```



```

SD.pig3 <- SD.pig3.save

#SD.pig3 <- SD.pig3/3
# generate random normal errors

Lim.eyes <-
as.integer(1000*c(1.05*max(VBeyes[,2]+2*SD.wetwteyes),0.95*min(VBeyes[,2]-
2*SD.wetwteyes)))/1000

Lim.pig3 <-
as.integer(1000*c(1.05*max(VBpig3[,2]+2*SD.pig3),0.95*min(VBpig3[,2]-
2*SD.pig3)))/1000

Lim.CL.f <-
as.integer(10*c(1.05*max(VBCL.f[,2]+2*SD.CL.f),0.95*min(VBCL.f[,2]-
2*SD.wetwteyes)))/10

Lim.CL.m <-
as.integer(10*c(1.05*max(VBCL.m[,2]+2*SD.CL.m),0.95*min(VBCL.m[,2]-
2*SD.wetwteyes)))/10

break.eyes <- c(0,(((seq(1,21)-1)/20)*(Lim.eyes[1]-Lim.eyes[2])+Lim.eyes[2]),
  Lim.eyes[1]*2)

break.pig3 <- c(0,(((seq(1,21)-1)/20)*(Lim.pig3[1]-Lim.pig3[2])+Lim.pig3[2]),
  Lim.pig3[1]*2)

break.pig3 <- as.integer(1000*break.pig3)/1000

break.CL.f <- c(0,(((seq(1,21)-1)/20)*(Lim.CL.f[1]-Lim.CL.f[2])+Lim.CL.f[2]),
  Lim.CL.f[1]*2)

break.CL.m <- c(0,(((seq(1,21)-1)/20)*(Lim.CL.m[1]-
Lim.CL.m[2])+Lim.CL.m[2]),
  Lim.CL.m[1]*2)

break.CL.f <- 10*as.integer(10*break.CL.f)/10
break.CL.m <- 10*as.integer(10*break.CL.m)/10

# set up full set of length bin labels
lev.e <- break.eyes
nL <- length(lev.e)
lev.ec <- character(length=nL)
for (r in 1:(nL-1)) {
  lev.ec[r] <- paste(paste("(",as.character(lev.e[r]),sep=""),
    paste(as.character(lev.e[r+1]),")",sep=""),sep=",")
}

```

```

lev.ec <- lev.ec [1:(nL-1)]

# set up full set of length bin labels
lev.p <- break.pig3
nL <- length(lev.p)
lev.pc <- character(length=nL)
for (r in 1:(nL-1)) {
  lev.pc[r] <- paste(paste("(",as.character(lev.p[r]),sep=""),
    paste(as.character(lev.p[r+1]),")",sep=""),sep=",")
}
lev.pc <- lev.pc [1:(nL-1)]

# set up full set of length bin labels
levCL.f <- break.CL.f
nL <- length(levCL.f)
levCL.fc <- character(length=nL)
for (r in 1:(nL-1)) {
  levCL.fc[r] <- paste(paste("(",as.character(levCL.f[r]),sep=""),
    paste(as.character(levCL.f[r+1]),")",sep=""),sep=",")
}
levCL.fc <- levCL.fc [1:(nL-1)]

# set up full set of length bin labels
levCL.m <- break.CL.m
nL <- length(levCL.m)
levCL.mc <- character(length=nL)
for (r in 1:(nL-1)) {
  levCL.mc[r] <- paste(paste("(",as.character(levCL.m[r]),sep=""),
    paste(as.character(levCL.m[r+1]),")",sep=""),sep=",")
}
levCL.mc <- levCL.mc [1:(nL-1)]

nL <- nL-1

Nlen <- nL

n.eyes.m <- matrix(data=rep(0,Nlen*No.ages), nrow=Nlen, ncol=No.ages)
n.pig3.m <- matrix(data=rep(0,Nlen*No.ages), nrow=Nlen, ncol=No.ages)
n.CL.f.m <- matrix(data=rep(0,Nlen*No.ages), nrow=Nlen, ncol=No.ages)
n.CL.m.m <- matrix(data=rep(0,Nlen*No.ages), nrow=Nlen, ncol=No.ages)

#r <- 1

for (r in 1:No.ages){
  wetwteyes.m[,r] <- rnorm(n=Nsim, mean=VBeyes[r,2], sd=SD.wetwteyes)

```

```

wetwteyes.m[,r] <- wetwteyes.m[,r]*as.integer(wetwteyes.m[,r]>0)
pig3.m[,r] <- rnorm(n=Nsim, mean=VBpig3[r,2], sd=SD.pig3)
pig3.m[,r] <- pig3.m[,r]*as.integer(pig3.m[,r]>0)
CL.f.m[,r] <- rnorm(n=Nsim, mean=10*VBCL.f[r,2], sd=10*SD.CL.f)
CL.f.m[,r] <- CL.f.m[,r]*as.integer(CL.f.m[,r]>0)
CL.m.m[,r] <- rnorm(n=Nsim, mean=10*VBCL.m[r,2], sd=10*SD.CL.m)
CL.m.m[,r] <- CL.m.m[,r]*as.integer(CL.m.m[,r]>0)

# aggregate into bins
eyes.f <- factor(x=cut(x=wetwteyes.m[,r], breaks=break.eyes))
pig3.f <- factor(x=cut(x=pig3.m[,r], breaks=break.pig3))
CL.f.f <- factor(x=cut(x=CL.f.m[,r], breaks=break.CL.f))
CL.m.f <- factor(x=cut(x=CL.m.m[,r], breaks=break.CL.m))

e.ind <- (seq(1,nL))[lev.ec %in% (levels(eyes.f))]
p.ind <- (seq(1,nL))[lev.pc %in% (levels(pig3.f))]
CL.f.ind <- (seq(1,nL))[levCL.fc %in% (levels(CL.f.f))]
CL.m.ind <- (seq(1,nL))[levCL.mc %in% (levels(CL.m.f))]

n.eyes.m[e.ind,r] <- as.vector(tapply(X=rep(1,Nsim), INDEX=eyes.f,
FUN=sum))
n.pig3.m[p.ind,r] <- as.vector(tapply(X=rep(1,Nsim), INDEX=pig3.f,
FUN=sum))
n.CL.f.m[CL.f.ind,r] <- as.vector(tapply(X=rep(1,Nsim), INDEX=CL.f.f,
FUN=sum))
n.CL.m.m[CL.m.ind,r] <- as.vector(tapply(X=rep(1,Nsim), INDEX=CL.m.f,
FUN=sum))

}

n.eyes.m
n.pig3.m
n.CL.f.m
n.CL.m.m

# analyse using continuation ratios

# set up predictors
break.eyes.m <- (break.eyes[c(1:(length(break.eyes)-
1))]+break.eyes[c(2:length(break.eyes))])/2
break.pig3.m <- (break.pig3[c(1:(length(break.pig3)-
1))]+break.pig3[c(2:length(break.pig3))])/2
break.CL.f.m <- (break.CL.f[c(1:(length(break.CL.f)-
1))]+break.CL.f[c(2:length(break.CL.f))])/2
break.CL.m.m <- (break.CL.m[c(1:(length(break.CL.m)-
1))]+break.CL.m[c(2:length(break.CL.m))])/2

# wet wt eyes

```

```

coef.m <- matrix(data=rep(0,3*2), nrow=3, ncol=2)
ndim <- length(break.eyes)-1
q.fit <- matrix(data=rep(0,4*ndim), nrow=ndim, ncol=4)
q.obs <- matrix(data=rep(0,4*ndim), nrow=ndim, ncol=4)
t.obs <- n.eyes.m %*% matrix(data=rep(1,4), nrow=4, ncol=1)
q.obs <- n.eyes.m/matrix(data=rep(t.obs,4), nrow=ndim, ncol=4)

for (r in 1:3) {
  #r <- 1
  nbin <- n.eyes.m[,r]
  nrem <- n.eyes.m[,c((r+1):4)] %*% matrix(data=rep(1,(4-r)), nrow=(4-r), ncol=1)
  data.df <- data.frame(nbin,nrem,break.eyes.m)
  glm.01 <- glm(cbind(nbin,nrem)~ break.eyes.m, family=binomial(link=logit),
  data=data.df)
  #summary(glm.01)
  coef.m[r,] <- glm.01$coef
  lp <- glm.01$coef[1]+glm.01$coef[2]*data.df$break.eyes.m
  fit.pr <- exp(lp)/(1+exp(lp))
  obs.pr <- nbin/(nbin+nrem)
  #plot(y=obs.pr, x=break.eyes.m, type="p")
  #lines(y=fit.pr, x=break.eyes.m)
  # unconditional probabilities
  if (r==1) q.fit[,r] <- fit.pr

  if (r>1) q.fit[,r] <- (1-q.fit[,seq(1,(r-1))])%*%matrix(data=rep(1,(r-1)), nrow=(r-1),
  ncol=1))*fit.pr
}

q.fit[,4] <- 1-q.fit[,seq(1,3)]%*%matrix(data=rep(1,3), nrow=3, ncol=1)

# calculate %deviance explained (Candy, 1991)

q.null.fit <- (t(q.obs *matrix(data=rep(t.obs,4), nrow=ndim, ncol=4))) %*%
matrix(data=rep(1,ndim), nrow=ndim, ncol=1)
q.null.fit <- q.null.fit/sum(q.null.fit)
q.null.fit.m <- matrix(data=rep(q.null.fit,ndim), nrow=ndim, ncol=4)
Dev.null <- -2*sum(as.vector(log(q.null.fit.m)*(q.obs *matrix(data=rep(t.obs,4),
nrow=ndim, ncol=4))))

Dev.fit <- -2*sum(as.vector(log(q.fit)*(q.obs *matrix(data=rep(t.obs,4),
nrow=ndim, ncol=4))))

Perc.dev <- 100*(Dev.null-Dev.fit)/Dev.null

print(c("%Dev explained",Perc.dev))

```

```

xyplot(q.obs[,1]+q.obs[,2]+q.obs[,3]+q.obs[,4]+q.fit[,1]+q.fit[,2]+q.fit[,3]+q.fit[,4]
~ break.eyes.m,
  xlab="Wet Weight Eyes (g)", ylab="Proportion", type="b",
  par.settings = list(superpose.line = list(lty = c(rep(0,4),c(1:4))),
col=c(rep(NA,4),c(1:4))),
  superpose.symbol= list(pch=c(c(1:4),rep(NA,4)),col=c(c(1:4),rep(NA,4)))),
  key=list(lines = list(lty=c(1:4), col=c(c(1:4),rep(NA,4))), points=list(pch=c(1:4),
col=c(1:4))),
  text = list(lab = c("12 month","24 month","36 month","48 month")), columns =
1,cex=0.6,
  x=0.75,y=0.95))

```

```

savePlot(filename = "Continuation regressions ages 1 to 4 wetwteyes.emf")

```

```

# pigment 3
coef.m <- matrix(data=rep(0,3*2), nrow=3, ncol=2)
ndim <- length(break.pig3)-1
q.fit <- matrix(data=rep(0,4*ndim), nrow=ndim, ncol=4)
q.obs <- matrix(data=rep(0,4*ndim), nrow=ndim, ncol=4)
t.obs <- n.pig3.m %%% matrix(data=rep(1,4), nrow=4, ncol=1)
q.obs <- n.pig3.m/matrix(data=rep(t.obs,4), nrow=ndim, ncol=4)

for (r in 1:3) {
#r <- 1
nbin <- n.pig3.m[,r]
nrem <- n.pig3.m[,c((r+1):4)] %%% matrix(data=rep(1,(4-r)), nrow=(4-r), ncol=1)
data.df <- data.frame(nbin,nrem,break.pig3.m)
glm.01 <- glm(cbind(nbin,nrem)~ break.pig3.m, family=binomial(link=logit),
data=data.df)
#summary(glm.01)
coef.m[r,] <- glm.01$coef
lp <- glm.01$coef[1]+glm.01$coef[2]*data.df$break.pig3.m
fit.pr <- exp(lp)/(1+exp(lp))
obs.pr <- nbin/(nbin+nrem)
#plot(y=obs.pr, x=break.pig3.m, type="p")
#lines(y=fit.pr, x=break.pig3.m)
# unconditional probabilities
if (r==1) q.fit[,r] <- fit.pr

if (r>1) q.fit[,r] <- (1-q.fit[,seq(1,(r-1))])%%matrix(data=rep(1,(r-1)), nrow=(r-1),
ncol=1))*fit.pr

}

```

```

q.fit[,4] <- 1-q.fit[,seq(1,3)]%%matrix(data=rep(1,3), nrow=3, ncol=1)

```

```

xyplot(q.obs[,1]+q.obs[,2]+q.obs[,3]+q.obs[,4]+q.fit[,1]+q.fit[,2]+q.fit[,3]+q.fit[,4]
~ break.pig3.m,

```

```

xlab="Pigment 3", ylab="Proportion", type="b",
par.settings = list(superpose.line = list(lty = c(rep(0,4),c(1:4))),
col=c(rep(NA,4),c(1:4))),
superpose.symbol= list(pch=c(c(1:4),rep(NA,4)),col=c(c(1:4),rep(NA,4))),
key=list(lines = list(lty=c(1:4), col=c(c(1:4),rep(NA,4))), points=list(pch=c(1:4),
col=c(1:4))),
text = list(lab = c("12 month","24 month","36 month","48 month")), columns =
1,cex=0.6,
x=0.75,y=0.95))

```

```

savePlot(filename = "Continuation regressions ages 1 to 4 pigment 3.emf")

```

```

q.null.fit <- (t(q.obs *matrix(data=rep(t.obs,4), nrow=ndim, ncol=4))) %%%
matrix(data=rep(1,ndim), nrow=ndim, ncol=1)
q.null.fit <- q.null.fit/sum(q.null.fit)
q.null.fit.m <- matrix(data=rep(q.null.fit,ndim), nrow=ndim, ncol=4)
Dev.null <- -2*sum(as.vector(log(q.null.fit.m)*(q.obs *matrix(data=rep(t.obs,4),
nrow=ndim, ncol=4))))

```

```

q.fit.v <- as.vector(q.fit)
Dev.fit <- -
2*sum(log(q.fit.v*as.integer(q.fit.v>0.0)+as.integer(q.fit.v==0))*as.vector((q.obs
*matrix(data=rep(t.obs,4), nrow=ndim, ncol=4))))

```

```

Perc.dev <- 100*(Dev.null-Dev.fit)/Dev.null

```

```

print(c("%Dev explained",Perc.dev))

```

```

# CL females

```

```

coef.m <- matrix(data=rep(0,3*2), nrow=3, ncol=2)
ndim <- length(break.CL.f)-1
q.fit <- matrix(data=rep(0,4*ndim), nrow=ndim, ncol=4)
q.obs <- matrix(data=rep(0,4*ndim), nrow=ndim, ncol=4)
t.obs <- n.CL.f.m %%% matrix(data=rep(1,4), nrow=4, ncol=1)
q.obs <- n.CL.f.m/matrix(data=rep(t.obs,4), nrow=ndim, ncol=4)

```

```

for (r in 1:3) {
#r <- 1
nbin <- n.CL.f.m[,r]
nrem <- n.CL.f.m[,c((r+1):4)] %%% matrix(data=rep(1,(4-r)), nrow=(4-r), ncol=1)
data.df <- data.frame(nbin,nrem,break.CL.f.m)
glm.01 <- glm(cbind(nbin,nrem)~ break.CL.f.m, family=binomial(link=logit),
data=data.df)
#summary(glm.01)
coef.m[r,] <- glm.01$coef
lp <- glm.01$coef[1]+glm.01$coef[2]*data.df$break.CL.f.m
fit.pr <- exp(lp)/(1+exp(lp))
obs.pr <- nbin/(nbin+nrem)
#plot(y=obs.pr, x=break.CL.f.m, type="p")

```

```

#lines(y=fit.pr, x=break.CL.f.m)
# unconditional probabilities
if (r==1) q.fit[,r] <- fit.pr

if (r>1) q.fit[,r] <- (1-q.fit[,seq(1,(r-1))])%*%matrix(data=rep(1,(r-1)), nrow=(r-1),
ncol=1))*fit.pr
}
q.fit[,4] <- 1-q.fit[,seq(1,3)]%*%matrix(data=rep(1,3), nrow=3, ncol=1)

xyplot(q.obs[,1]+q.obs[,2]+q.obs[,3]+q.obs[,4]+q.fit[,1]+q.fit[,2]+q.fit[,3]+q.fit[,4]
~ break.CL.f.m,
  xlab="CL females (mm)", ylab="Proportion", type="b",
  par.settings = list(superpose.line = list(lty = c(rep(0,4),c(1:4))),
col=c(rep(NA,4),c(1:4))),
  superpose.symbol= list(pch=c(c(1:4),rep(NA,4)),col=c(c(1:4),rep(NA,4)))),
  key=list(lines = list(lty=c(1:4), col=c(c(1:4),rep(NA,4))), points=list(pch=c(1:4),
col=c(1:4))),
  text = list(lab = c("12 month", "24 month", "36 month", "48 month")), columns =
1,cex=0.6,
  x=0.75,y=0.95))

savePlot(filename = "Continuation regressions ages 1 to 4 CL females.emf")

q.null.fit <- (t(q.obs *matrix(data=rep(t.obs,4), nrow=ndim, ncol=4))) %*%
matrix(data=rep(1,ndim), nrow=ndim, ncol=1)
q.null.fit <- q.null.fit/sum(q.null.fit)
q.null.fit.m <- matrix(data=rep(q.null.fit,ndim), nrow=ndim, ncol=4)
Dev.null <- -2*sum(as.vector(log(q.null.fit.m))*(q.obs *matrix(data=rep(t.obs,4),
nrow=ndim, ncol=4))))

Dev.fit <- -2*sum(as.vector(log(q.fit)*(q.obs *matrix(data=rep(t.obs,4),
nrow=ndim, ncol=4))))

Perc.dev <- 100*(Dev.null-Dev.fit)/Dev.null

print(c("%Dev explained",Perc.dev))

# CL males
coef.m <- matrix(data=rep(0,3*2), nrow=3, ncol=2)
ndim <- length(break.CL.m)-1
q.fit <- matrix(data=rep(0,4*ndim), nrow=ndim, ncol=4)
q.obs <- matrix(data=rep(0,4*ndim), nrow=ndim, ncol=4)
t.obs <- n.CL.m.m %*% matrix(data=rep(1,4), nrow=4, ncol=1)
q.obs <- n.CL.m.m/matrix(data=rep(t.obs,4), nrow=ndim, ncol=4)

for (r in 1:3) {
#r <- 1
nbin <- n.CL.m.m[,r]

```

```

nrem <- n.CL.m.m[,c((r+1):4)] %%% matrix(data=rep(1,(4-r)), nrow=(4-r),
ncol=1)
data.df <- data.frame(nbin,nrem,break.CL.m.m)
glm.01 <- glm(cbind(nbin,nrem)~ break.CL.m.m, family=binomial(link=logit),
data=data.df)
#summary(glm.01)
coef.m[r,] <- glm.01$coef
lp <- glm.01$coef[1]+glm.01$coef[2]*data.df$break.CL.m.m
fit.pr <- exp(lp)/(1+exp(lp))
obs.pr <- nbin/(nbin+nrem)
#plot(y=obs.pr, x=break.CL.m.m, type="p")
#lines(y=fit.pr, x=break.CL.m.m)
# unconditional probabilities
if (r==1) q.fit[,r] <- fit.pr

if (r>1) q.fit[,r] <- (1-q.fit[,seq(1,(r-1))])%%matrix(data=rep(1,(r-1)), nrow=(r-1),
ncol=1))*fit.pr
}
q.fit[,4] <- 1-q.fit[,seq(1,3)]%%matrix(data=rep(1,3), nrow=3, ncol=1)

xyplot(q.obs[,1]+q.obs[,2]+q.obs[,3]+q.obs[,4]+q.fit[,1]+q.fit[,2]+q.fit[,3]+q.fit[,4]
~ break.CL.m.m,
xlab="CL males (mm)", ylab="Proportion", type="b",
par.settings = list(superpose.line = list(lty = c(rep(0,4),c(1:4))),
col=c(rep(NA,4),c(1:4))),
superpose.symbol= list(pch=c(c(1:4),rep(NA,4)),col=c(c(1:4),rep(NA,4)))),
key=list(lines = list(lty=c(1:4), col=c(c(1:4),rep(NA,4))), points=list(pch=c(1:4),
col=c(1:4))),
text = list(lab = c("12 month","24 month","36 month","48 month")), columns =
1,cex=0.6,
x=0.75,y=0.95))

savePlot(filename = "Continuation regressions ages 1 to 4 CL males.emf")

q.null.fit <- (t(q.obs *matrix(data=rep(t.obs,4), nrow=ndim, ncol=4))) %%%
matrix(data=rep(1,ndim), nrow=ndim, ncol=1)
q.null.fit <- q.null.fit/sum(q.null.fit)
q.null.fit.m <- matrix(data=rep(q.null.fit,ndim), nrow=ndim, ncol=4)
Dev.null <- -2*sum(as.vector(log(q.null.fit.m)*(q.obs *matrix(data=rep(t.obs,4),
nrow=ndim, ncol=4))))

Dev.fit <- -2*sum(as.vector(log(q.fit)*(q.obs *matrix(data=rep(t.obs,4),
nrow=ndim, ncol=4))))

Perc.dev <- 100*(Dev.null-Dev.fit)/Dev.null

print(c("%Dev explained",Perc.dev))
Output

```



```

xyplot(q.obs[,1]+q.obs[,2]+q.obs[,3]+q.obs[,4]+q.fit[,1]+q.fit[,2]+q.fit[,3]+q.fit[,4]
~ break.pig3.m,
+   xlab="Pigment 3", ylab="Proportion", type="b",
+   par.settings = list(superpose.line = list(lty = c(rep(0,4),c(1:4))),
col=c(rep(NA,4),c(1:4))),
+   superpose.symbol= list(pch=c(c(1:4),rep(NA,4)),col=c(c(1:4),rep(NA,4))),
+   key=list(lines = list(lty=c(1:4), col=c(c(1:4),rep(NA,4))),
points=list(pch=c(1:4), col=c(1:4))),
+   text = list(lab = c("12 month", "24 month", "36 month", "48 month")), columns
= 1,cex=0.6,
+   x=0.75,y=0.75))
>
> savePlot(filename = "Continuation regressions ages 1 to 4 pigment 3.emf")
>
> q.null.fit <- (t(q.obs *matrix(data=rep(t.obs,4), nrow=ndim, ncol=4))) %*%
matrix(data=rep(1,ndim), nrow=ndim, ncol=1)
> q.null.fit <- q.null.fit/sum(q.null.fit)
> q.null.fit.m <- matrix(data=rep(q.null.fit,ndim), nrow=ndim, ncol=4)
> Dev.null <- -2*sum(as.vector(log(q.null.fit.m)*(q.obs *matrix(data=rep(t.obs,4),
nrow=ndim, ncol=4))))
>
> q.fit.v <- as.vector(q.fit)
> Dev.fit <- -
2*sum(log(q.fit.v*as.integer(q.fit.v>0.0)+as.integer(q.fit.v==0))*as.vector((q.obs
*matrix(data=rep(t.obs,4), nrow=ndim, ncol=4))))
>
> Perc.dev <- 100*(Dev.null-Dev.fit)/Dev.null
>
> print(c("%Dev explained",Perc.dev))
[1] "%Dev explained" "22.4965309107160"
>
>
xyplot(q.obs[,1]+q.obs[,2]+q.obs[,3]+q.obs[,4]+q.fit[,1]+q.fit[,2]+q.fit[,3]+q.fit[,4]
~ break.pig3.m,
+   xlab="Pigment 3", ylab="Proportion", type="b",
+   par.settings = list(superpose.line = list(lty = c(rep(0,4),c(1:4))),
col=c(rep(NA,4),c(1:4))),
+   superpose.symbol= list(pch=c(c(1:4),rep(NA,4)),col=c(c(1:4),rep(NA,4))),
+   key=list(lines = list(lty=c(1:4), col=c(c(1:4),rep(NA,4))),
points=list(pch=c(1:4), col=c(1:4))),
+   text = list(lab = c("12 month", "24 month", "36 month", "48 month")), columns
= 1,cex=0.6,
+   x=0.75,y=0.95))
>
> savePlot(filename = "Continuation regressions ages 1 to 4 pigment 3.emf")
>
> Lim.eyes <-
as.integer(1000*c(1.05*max(VBeyes[,2]+2*SD.wetwteyes),0.95*min(VBeyes[,2]-
2*SD.wetwteyes)))/1000
>

```

```

> Lim.pig3 <-
as.integer(1000*c(1.05*max(VBpig3[,2]+2*SD.pig3),0.95*min(VBpig3[,2]-
2*SD.pig3)))/1000
>
> Lim.CL.f <-
as.integer(10*c(1.05*max(VBCL.f[,2]+2*SD.CL.f),0.95*min(VBCL.f[,2]-
2*SD.wetwteyes)))/10
>
> Lim.CL.m <-
as.integer(10*c(1.05*max(VBCL.m[,2]+2*SD.CL.m),0.95*min(VBCL.m[,2]-
2*SD.wetwteyes)))/10
>
>
> break.eyes <- c(0,(((seq(1,21)-1)/20)*(Lim.eyes[1]-Lim.eyes[2])+Lim.eyes[2]),
+   Lim.eyes[1]*2)
>
> break.pig3 <- c(0,(((seq(1,21)-1)/20)*(Lim.pig3[1]-Lim.pig3[2])+Lim.pig3[2]),
+   Lim.pig3[1]*2)
>
> break.pig3 <- as.integer(1000*break.pig3)/1000
>
> break.CL.f <- c(0,(((seq(1,21)-1)/20)*(Lim.CL.f[1]-Lim.CL.f[2])+Lim.CL.f[2]),
+   Lim.CL.f[1]*2)
>
> break.CL.m <- c(0,(((seq(1,21)-1)/20)*(Lim.CL.m[1]-
Lim.CL.m[2])+Lim.CL.m[2]),
+   Lim.CL.m[1]*2)
>
> break.CL.f <- 10*as.integer(10*break.CL.f)/10
> break.CL.m <- 10*as.integer(10*break.CL.m)/10
>
>
> # set up full set of length bin labels
> lev.e <- break.eyes
> nL <- length(lev.e)
> lev.ec <- character(length=nL)
> for (r in 1:(nL-1)) {
+   lev.ec[r] <- paste(paste("(",as.character(lev.e[r]),sep=""),
+     paste(as.character(lev.e[r+1]),")",sep=""),sep=",")
+ }
> lev.ec <- lev.ec [1:(nL-1)]
>
> # set up full set of length bin labels
> lev.p <- break.pig3
> nL <- length(lev.p)
> lev.pc <- character(length=nL)
> for (r in 1:(nL-1)) {
+   lev.pc[r] <- paste(paste("(",as.character(lev.p[r]),sep=""),
+     paste(as.character(lev.p[r+1]),")",sep=""),sep=",")
+ }

```

```

> lev.pc <- lev.pc [1:(nL-1)]
>
> # set up full set of length bin labels
> levCL.f <- break.CL.f
> nL <- length(levCL.f)
> levCL.fc <- character(length=nL)
> for (r in 1:(nL-1)) {
+   levCL.fc[r] <- paste(paste("(",as.character(levCL.f[r]),sep=""),
+     paste(as.character(levCL.f[r+1]),")",sep=""),sep=",")
+ }
> levCL.fc <- levCL.fc [1:(nL-1)]
>
> # set up full set of length bin labels
> levCL.m <- break.CL.m
> nL <- length(levCL.m)
> levCL.mc <- character(length=nL)
> for (r in 1:(nL-1)) {
+   levCL.mc[r] <- paste(paste("(",as.character(levCL.m[r]),sep=""),
+     paste(as.character(levCL.m[r+1]),")",sep=""),sep=",")
+ }
> levCL.mc <- levCL.mc [1:(nL-1)]
>
>
>
> nL <- nL-1
>
> Nlen <- nL
>
> n.eyes.m <- matrix(data=rep(0,Nlen*No.ages), nrow=Nlen, ncol=No.ages)
>
> n.pig3.m <- matrix(data=rep(0,Nlen*No.ages), nrow=Nlen, ncol=No.ages)
>
> n.CL.f.m <- matrix(data=rep(0,Nlen*No.ages), nrow=Nlen, ncol=No.ages)
>
> n.CL.m.m <- matrix(data=rep(0,Nlen*No.ages), nrow=Nlen, ncol=No.ages)
>
>
>
> #r <- 1
>
> for (r in 1:No.ages){
+   wetwteyes.m[,r] <- rmorm(n=Nsim, mean=VBeyes[r,2], sd=SD.wetwteyes)
+   wetwteyes.m[,r] <- wetwteyes.m[,r]*as.integer(wetwteyes.m[,r]>0)
+   pig3.m[,r] <- rmorm(n=Nsim, mean=VBpig3[r,2], sd=SD.pig3)
+   pig3.m[,r] <- pig3.m[,r]*as.integer(pig3.m[,r]>0)
+   CL.f.m[,r] <- rmorm(n=Nsim, mean=10*VBCL.f[r,2], sd=10*SD.CL.f)
+   CL.f.m[,r] <- CL.f.m[,r]*as.integer(CL.f.m[,r]>0)
+   CL.m.m[,r] <- rmorm(n=Nsim, mean=10*VBCL.m[r,2], sd=10*SD.CL.m)
+   CL.m.m[,r] <- CL.m.m[,r]*as.integer(CL.m.m[,r]>0)
+ }

```

```

+
+
+ # aggregate into bins
+ eyes.f <- factor(x=cut(x=wetwteyes.m[,r], breaks=break.eyes))
+ pig3.f <- factor(x=cut(x=pig3.m[,r], breaks=break.pig3))
+ CL.f.f <- factor(x=cut(x=CL.f.m[,r], breaks=break.CL.f))
+ CL.m.f <- factor(x=cut(x=CL.m.m[,r], breaks=break.CL.m))
+
+ e.ind <- (seq(1,nL))[lev.ec %in% (levels(eyes.f))]
+ p.ind <- (seq(1,nL))[lev.pc %in% (levels(pig3.f))]
+ CL.f.ind <- (seq(1,nL))[levCL.fc %in% (levels(CL.f.f))]
+ CL.m.ind <- (seq(1,nL))[levCL.mc %in% (levels(CL.m.f))]
+
+ n.eyes.m[e.ind,r] <- as.vector(tapply(X=rep(1,Nsim), INDEX=eyes.f,
FUN=sum))
+ n.pig3.m[p.ind,r] <- as.vector(tapply(X=rep(1,Nsim), INDEX=pig3.f,
FUN=sum))
+ n.CL.f.m[CL.f.ind,r] <- as.vector(tapply(X=rep(1,Nsim), INDEX=CL.f.f,
FUN=sum))
+ n.CL.m.m[CL.m.ind,r] <- as.vector(tapply(X=rep(1,Nsim), INDEX=CL.m.f,
FUN=sum))
+
+ }
>
> n.eyes.m
      [,1] [,2] [,3] [,4]
[1,]  17   0   0   0
[2,]  13   0   0   0
[3,]  46   2   0   1
[4,]  53   3   1   0
[5,]  80  12   0   0
[6,]  79  16   5   3
[7,] 124  31  10   7
[8,] 131  51  14   6
[9,] 114  75  37  22
[10,]  99 114  54  35
[11,]  92 120  60  58
[12,]  59 124 117  89
[13,]  39 109 115  98
[14,]  26 116  94 128
[15,]  17  82 141 135
[16,]   6  56 108 122
[17,]   2  44  92 105
[18,]   2  21  68  73
[19,]   0  14  38  47
[20,]   0   7  22  27
[21,]   0   2  15  27
[22,]   0   1   9  17
> n.pig3.m
      [,1] [,2] [,3] [,4]

```

```

[1,] 13 0 0 0
[2,] 21 2 0 0
[3,] 41 4 0 0
[4,] 46 5 1 0
[5,] 93 17 2 1
[6,] 133 22 7 2
[7,] 123 33 7 3
[8,] 145 69 18 11
[9,] 120 81 37 22
[10,] 99 107 45 41
[11,] 72 124 107 57
[12,] 44 139 118 79
[13,] 27 127 140 111
[14,] 16 103 136 117
[15,] 4 74 132 150
[16,] 0 43 105 148
[17,] 0 31 60 100
[18,] 1 10 41 67
[19,] 0 5 24 48
[20,] 0 1 11 18
[21,] 0 2 6 9
[22,] 0 1 3 16

```

> n.CL.f.m

```

[,1][,2][,3][,4]
[1,] 268 35 24 21
[2,] 83 21 7 18
[3,] 106 22 23 24
[4,] 92 41 29 23
[5,] 86 38 41 30
[6,] 88 58 49 63
[7,] 61 66 68 74
[8,] 58 77 63 84
[9,] 46 86 99 85
[10,] 35 98 86 91
[11,] 40 147 123 129
[12,] 11 70 96 75
[13,] 13 72 71 66
[14,] 6 58 60 57
[15,] 1 33 54 38
[16,] 2 24 32 45
[17,] 0 23 27 27
[18,] 2 15 11 19
[19,] 1 8 16 10
[20,] 0 2 11 8
[21,] 1 5 6 9
[22,] 0 1 4 4

```

> n.CL.m.m

```

[,1][,2][,3][,4]
[1,] 326 16 13 9
[2,] 57 11 4 8

```

```

[3,] 118 19 10 7
[4,] 103 28 13 17
[5,] 91 57 40 36
[6,] 76 74 43 43
[7,] 79 94 77 56
[8,] 58 73 81 80
[9,] 34 98 90 80
[10,] 15 121 113 106
[11,] 17 118 107 102
[12,] 9 50 60 68
[13,] 6 67 82 96
[14,] 7 61 71 84
[15,] 0 41 55 69
[16,] 1 31 49 52
[17,] 2 16 34 32
[18,] 0 12 27 27
[19,] 1 10 16 13
[20,] 0 2 8 6
[21,] 0 1 1 3
[22,] 0 0 6 6
>
> # analyse using continuation ratios
>
> # set up predictors
> break.eyes.m <- (break.eyes[c(1:(length(break.eyes)-
1))]+break.eyes[c(2:length(break.eyes))])/2
> break.pig3.m <- (break.pig3[c(1:(length(break.pig3)-
1))]+break.pig3[c(2:length(break.pig3))])/2
> break.CL.f.m <- (break.CL.f[c(1:(length(break.CL.f)-
1))]+break.CL.f[c(2:length(break.CL.f))])/2
> break.CL.m.m <- (break.CL.m[c(1:(length(break.CL.m)-
1))]+break.CL.m[c(2:length(break.CL.m))])/2
>
> # wet wt eyes
> coef.m <- matrix(data=rep(0,3*2), nrow=3, ncol=2)
> ndim <- length(break.eyes)-1
> q.fit <- matrix(data=rep(0,4*ndim), nrow=ndim, ncol=4)
> q.obs <- matrix(data=rep(0,4*ndim), nrow=ndim, ncol=4)
> t.obs <- n.eyes.m %*% matrix(data=rep(1,4), nrow=4, ncol=1)
> q.obs <- n.eyes.m/matrix(data=rep(t.obs,4), nrow=ndim, ncol=4)
>
>
> for (r in 1:3) {
+ #r <- 1
+ nbin <- n.eyes.m[,r]
+ nrem <- n.eyes.m[,c((r+1):4)] %*% matrix(data=rep(1,(4-r)), nrow=(4-r),
ncol=1)
+ data.df <- data.frame(nbin,nrem,break.eyes.m)
+ glm.01 <- glm(cbind(nbin,nrem)~ break.eyes.m, family=binomial(link=logit),
data=data.df)

```

```

+ #summary(glm.01)
+ coef.m[r,] <- glm.01$coef
+ lp <- glm.01$coef[1]+glm.01$coef[2]*data.df$break.eyes.m
+ fit.pr <- exp(lp)/(1+exp(lp))
+ obs.pr <- nbin/(nbin+nrem)
+ #plot(y=obs.pr, x=break.eyes.m, type="p")
+ #lines(y=fit.pr, x=break.eyes.m)
+ # unconditional probabilities
+ if (r==1) q.fit[,r] <- fit.pr
+
+ if (r>1) q.fit[,r] <- (1-q.fit[,seq(1,(r-1))])%*%matrix(data=rep(1,(r-1)), nrow=(r-1), ncol=1))*fit.pr
+
+ }
>
> q.fit[,4] <- 1-q.fit[,seq(1,3)]%*%matrix(data=rep(1,3), nrow=3, ncol=1)
>
> # calculate %deviance explained (Candy, 1991)
>
> q.null.fit <- (t(q.obs *matrix(data=rep(t.obs,4), nrow=ndim, ncol=4))) %*%
matrix(data=rep(1,ndim), nrow=ndim, ncol=1)
> q.null.fit <- q.null.fit/sum(q.null.fit)
> q.null.fit.m <- matrix(data=rep(q.null.fit,ndim), nrow=ndim, ncol=4)
> Dev.null <- -2*sum(as.vector(log(q.null.fit.m)*(q.obs *matrix(data=rep(t.obs,4),
nrow=ndim, ncol=4))))
>
> Dev.fit <- -2*sum(as.vector(log(q.fit)*(q.obs *matrix(data=rep(t.obs,4),
nrow=ndim, ncol=4))))
>
> Perc.dev <- 100*(Dev.null-Dev.fit)/Dev.null
>
> print(c("%Dev explained",Perc.dev))
[1] "%Dev explained" "18.4522167982732"
>
>
>
xyplot(q.obs[,1]+q.obs[,2]+q.obs[,3]+q.obs[,4]+q.fit[,1]+q.fit[,2]+q.fit[,3]+q.fit[,4]
~ break.eyes.m,
+ xlab="Wet Weight Eyes (g)", ylab="Proportion", type="b",
+ par.settings = list(superpose.line = list(lty = c(rep(0,4),c(1:4)),
col=c(rep(NA,4),c(1:4))),
+ superpose.symbol= list(pch=c(c(1:4),rep(NA,4)),col=c(c(1:4),rep(NA,4)))),
+ key=list(lines = list(lty=c(1:4), col=c(c(1:4),rep(NA,4))),
points=list(pch=c(1:4), col=c(c(1:4))),
+ text = list(lab = c("12 month","24 month","36 month","48 month")), columns
= 1,cex=0.6,
+ x=0.75,y=0.95))
>
> savePlot(filename = "Continuation regressions ages 1 to 4 wetwteyes.emf")
>

```

```

>
>
> # pigment 3
> coef.m <- matrix(data=rep(0,3*2), nrow=3, ncol=2)
> ndim <- length(break.pig3)-1
> q.fit <- matrix(data=rep(0,4*ndim), nrow=ndim, ncol=4)
> q.obs <- matrix(data=rep(0,4*ndim), nrow=ndim, ncol=4)
> t.obs <- n.pig3.m %%% matrix(data=rep(1,4), nrow=4, ncol=1)
> q.obs <- n.pig3.m/matrix(data=rep(t.obs,4), nrow=ndim, ncol=4)
>
>
> for (r in 1:3) {
+ #r <- 1
+ nbin <- n.pig3.m[,r]
+ nrem <- n.pig3.m[,c((r+1):4)] %%% matrix(data=rep(1,(4-r)), nrow=(4-r),
ncol=1)
+ data.df <- data.frame(nbin,nrem,break.pig3.m)
+ glm.01 <- glm(cbind(nbin,nrem)~ break.pig3.m, family=binomial(link=logit),
data=data.df)
+ #summary(glm.01)
+ coef.m[r,] <- glm.01$coef
+ lp <- glm.01$coef[1]+glm.01$coef[2]*data.df$break.pig3.m
+ fit.pr <- exp(lp)/(1+exp(lp))
+ obs.pr <- nbin/(nbin+nrem)
+ #plot(y=obs.pr, x=break.pig3.m, type="p")
+ #lines(y=fit.pr, x=break.pig3.m)
+ # unconditional probabilities
+ if (r==1) q.fit[,r] <- fit.pr
+
+ if (r>1) q.fit[,r] <- (1-q.fit[,seq(1,(r-1))])%%matrix(data=rep(1,(r-1)), nrow=(r-
1), ncol=1))*fit.pr
+
+ }
>
> q.fit[,4] <- 1-q.fit[,seq(1,3)]%%matrix(data=rep(1,3), nrow=3, ncol=1)
>
>
xyplot(q.obs[,1]+q.obs[,2]+q.obs[,3]+q.obs[,4]+q.fit[,1]+q.fit[,2]+q.fit[,3]+q.fit[,4]
~ break.pig3.m,
+ xlab="Pigment 3", ylab="Proportion", type="b",
+ par.settings = list(superpose.line = list(lty = c(rep(0,4),c(1:4))),
col=c(rep(NA,4),c(1:4))),
+ superpose.symbol= list(pch=c(c(1:4),rep(NA,4)),col=c(c(1:4),rep(NA,4)))),
+ key=list(lines = list(lty=c(1:4), col=c(c(1:4),rep(NA,4))),
points=list(pch=c(1:4), col=c(1:4))),
+ text = list(lab = c("12 month","24 month","36 month","48 month")), columns
= 1,cex=0.6,
+ x=0.75,y=0.95))
>
> savePlot(filename = "Continuation regressions ages 1 to 4 pigment 3.emf")

```



```

>
> q.null.fit <- (t(q.obs * matrix(data=rep(t.obs,4), nrow=ndim, ncol=4))) %*%
matrix(data=rep(1,ndim), nrow=ndim, ncol=1)
> q.null.fit <- q.null.fit/sum(q.null.fit)
> q.null.fit.m <- matrix(data=rep(q.null.fit,ndim), nrow=ndim, ncol=4)
> Dev.null <- -2*sum(as.vector(log(q.null.fit.m)*(q.obs * matrix(data=rep(t.obs,4),
nrow=ndim, ncol=4))))
>
> q.fit.v <- as.vector(q.fit)
> Dev.fit <- -
2*sum(log(q.fit.v*as.integer(q.fit.v>0.0)+as.integer(q.fit.v==0))*as.vector((q.obs
* matrix(data=rep(t.obs,4), nrow=ndim, ncol=4))))
>
> Perc.dev <- 100*(Dev.null-Dev.fit)/Dev.null
>
> print(c("%Dev explained",Perc.dev))
[1] "%Dev explained" "21.4008513033684"
>
> # CL females
> coef.m <- matrix(data=rep(0,3*2), nrow=3, ncol=2)
> ndim <- length(break.CL.f)-1
> q.fit <- matrix(data=rep(0,4*ndim), nrow=ndim, ncol=4)
> q.obs <- matrix(data=rep(0,4*ndim), nrow=ndim, ncol=4)
> t.obs <- n.CL.f.m %*% matrix(data=rep(1,4), nrow=4, ncol=1)
> q.obs <- n.CL.f.m/matrix(data=rep(t.obs,4), nrow=ndim, ncol=4)
>
>
> for (r in 1:3) {
+ #r <- 1
+ nbin <- n.CL.f.m[,r]
+ nrem <- n.CL.f.m[,c((r+1):4)] %*% matrix(data=rep(1,(4-r)), nrow=(4-r),
ncol=1)
+ data.df <- data.frame(nbin,nrem,break.CL.f.m)
+ glm.01 <- glm(cbind(nbin,nrem)~ break.CL.f.m, family=binomial(link=logit),
data=data.df)
+ #summary(glm.01)
+ coef.m[r,] <- glm.01$coef
+ lp <- glm.01$coef[1]+glm.01$coef[2]*data.df$break.CL.f.m
+ fit.pr <- exp(lp)/(1+exp(lp))
+ obs.pr <- nbin/(nbin+nrem)
+ #plot(y=obs.pr, x=break.CL.f.m, type="p")
+ #lines(y=fit.pr, x=break.CL.f.m)
+ # unconditional probabilities
+ if (r==1) q.fit[,r] <- fit.pr
+
+ if (r>1) q.fit[,r] <- (1-q.fit[,seq(1,(r-1))])%*%matrix(data=rep(1,(r-1)), nrow=(r-
1), ncol=1))*fit.pr
+
+ }
>

```

```

> q.fit[,4] <- 1-q.fit[,seq(1,3)]%*%matrix(data=rep(1,3), nrow=3, ncol=1)
>
>
xyplot(q.obs[,1]+q.obs[,2]+q.obs[,3]+q.obs[,4]+q.fit[,1]+q.fit[,2]+q.fit[,3]+q.fit[,4]
~ break.CL.f.m,
+   xlab="CL females (mm)", ylab="Proportion", type="b",
+   par.settings = list(superpose.line = list(lty = c(rep(0,4),c(1:4))),
col=c(rep(NA,4),c(1:4))),
+   superpose.symbol= list(pch=c(c(1:4),rep(NA,4)),col=c(c(1:4),rep(NA,4)))),
+   key=list(lines = list(lty=c(1:4), col=c(c(1:4),rep(NA,4))),
points=list(pch=c(1:4), col=c(1:4))),
+   text = list(lab = c("12 month","24 month","36 month","48 month")), columns
= 1,cex=0.6,
+   x=0.75,y=0.95))
>
> savePlot(filename = "Continuation regressions ages 1 to 4 CL females.emf")
>
> q.null.fit <- (t(q.obs *matrix(data=rep(t.obs,4), nrow=ndim, ncol=4))) %*%
matrix(data=rep(1,ndim), nrow=ndim, ncol=1)
> q.null.fit <- q.null.fit/sum(q.null.fit)
> q.null.fit.m <- matrix(data=rep(q.null.fit,ndim), nrow=ndim, ncol=4)
> Dev.null <- -2*sum(as.vector(log(q.null.fit.m))*(q.obs *matrix(data=rep(t.obs,4),
nrow=ndim, ncol=4))))
>
> Dev.fit <- -2*sum(as.vector(log(q.fit))*(q.obs *matrix(data=rep(t.obs,4),
nrow=ndim, ncol=4))))
>
> Perc.dev <- 100*(Dev.null-Dev.fit)/Dev.null
>
> print(c("%Dev explained",Perc.dev))
[1] "%Dev explained" "8.27842786814089"
>
> # CL males
> coef.m <- matrix(data=rep(0,3*2), nrow=3, ncol=2)
> ndim <- length(break.CL.m)-1
> q.fit <- matrix(data=rep(0,4*ndim), nrow=ndim, ncol=4)
> q.obs <- matrix(data=rep(0,4*ndim), nrow=ndim, ncol=4)
> t.obs <- n.CL.m.m %*% matrix(data=rep(1,4), nrow=4, ncol=1)
> q.obs <- n.CL.m.m/matrix(data=rep(t.obs,4), nrow=ndim, ncol=4)
>
>
> for (r in 1:3) {
+ #r <- 1
+ nbin <- n.CL.m.m[,r]
+ nrem <- n.CL.m.m[,c((r+1):4)] %*% matrix(data=rep(1,(4-r)), nrow=(4-r),
ncol=1)
+ data.df <- data.frame(nbin,nrem,break.CL.m.m)
+ glm.01 <- glm(cbind(nbin,nrem)~ break.CL.m.m, family=binomial(link=logit),
data=data.df)
+ #summary(glm.01)

```

```

+ coef.m[r,] <- glm.01$coef
+ lp <- glm.01$coef[1]+glm.01$coef[2]*data.df$break.CL.m.m
+ fit.pr <- exp(lp)/(1+exp(lp))
+ obs.pr <- nbin/(nbin+nrem)
+ #plot(y=obs.pr, x=break.CL.m.m, type="p")
+ #lines(y=fit.pr, x=break.CL.m.m)
+ # unconditional probabilities
+ if (r==1) q.fit[,r] <- fit.pr
+
+ if (r>1) q.fit[,r] <- (1-q.fit[,seq(1,(r-1))])%*%matrix(data=rep(1,(r-1)), nrow=(r-1), ncol=1))*fit.pr
+
+ }
>
> q.fit[,4] <- 1-q.fit[,seq(1,3)]%*%matrix(data=rep(1,3), nrow=3, ncol=1)
>
>
xyplot(q.obs[,1]+q.obs[,2]+q.obs[,3]+q.obs[,4]+q.fit[,1]+q.fit[,2]+q.fit[,3]+q.fit[,4]
~ break.CL.m.m,
+   xlab="CL males (mm)", ylab="Proportion", type="b",
+   par.settings = list(superpose.line = list(lty = c(rep(0,4),c(1:4))),
col=c(rep(NA,4),c(1:4))),
+   superpose.symbol= list(pch=c(c(1:4),rep(NA,4)),col=c(c(1:4),rep(NA,4))),
+   key=list(lines = list(lty=c(1:4), col=c(c(1:4),rep(NA,4))),
points=list(pch=c(1:4), col=c(1:4))),
+   text = list(lab = c("12 month","24 month","36 month","48 month")), columns
= 1,cex=0.6,
+   x=0.75,y=0.95))
>
> savePlot(filename = "Continuation regressions ages 1 to 4 CL males.emf")
>
> q.null.fit <- (t(q.obs *matrix(data=rep(t.obs,4), nrow=ndim, ncol=4))) %*%
matrix(data=rep(1,ndim), nrow=ndim, ncol=1)
> q.null.fit <- q.null.fit/sum(q.null.fit)
> q.null.fit.m <- matrix(data=rep(q.null.fit,ndim), nrow=ndim, ncol=4)
> Dev.null <- -2*sum(as.vector(log(q.null.fit.m)*(q.obs *matrix(data=rep(t.obs,4),
nrow=ndim, ncol=4))))
>
> Dev.fit <- -2*sum(as.vector(log(q.fit)*(q.obs *matrix(data=rep(t.obs,4),
nrow=ndim, ncol=4))))
>
> Perc.dev <- 100*(Dev.null-Dev.fit)/Dev.null
>
> print(c("%Dev explained",Perc.dev))
[1] "%Dev explained" "14.4294799493693"

```

## Appendix III: Logistic discriminant analysis

---

### *Code in R*

```
# Angela's pigment analyses
# part 1 Cumulative pigment vs age

# run function "plot.trellis.sebar" at end of this script first

library(lattice)

data.df <- read.csv(file="Part1.csv")

summary(data.df)

data.mf.df <- data.df[data.df$sex!="j",]

data.mf.df$sex <- as.factor(as.character(data.mf.df$sex))

xyplot(pigment.3 ~ Age_mth | sex, data=data.mf.df, type="p")

xyplot(CL ~ Age_mth | sex, data=data.mf.df, type="p")

xyplot(CL ~ pigment.3 | sex, data=data.mf.df, type="p")

xyplot(pigment.1 ~ wetwteyes | sex, data=data.mf.df, type="p")

xyplot(pigment.3 ~ wetwteyes | sex, data=data.mf.df, type="p")

xyplot(wetwteyes ~ pigment.3 | sex, data=data.mf.df, type="p")

xyplot(wetwteyes ~ Age_mth | sex, data=data.mf.df, type="p")

mean.CL <-
as.vector(tapply(data.mf.df$CL,INDEX=list(data.mf.df$age.f,data.mf.df$sex),
FUN=mean))
SD.CL <-
(as.vector(tapply(data.mf.df$CL,INDEX=list(data.mf.df$age.f,data.mf.df$sex),
FUN=var)))^0.5
N.CL <-
as.vector(tapply(rep(1,length(data.mf.df$CL)),INDEX=list(data.mf.df$age.f,data.
mf.df$sex), FUN=sum))

mean.wetwteyes <-
as.vector(tapply(data.mf.df$wetwteyes,INDEX=list(data.mf.df$age.f,data.mf.df$sex),
FUN=mean))
```

```

SD.wetwteyes <-
(as.vector(tapply(data.mf.df$wetwteyes,INDEX=list(data.mf.df$age.f,data.mf.df$sex), FUN=var)))^0.5
N.wetwteyes <-
as.vector(tapply(rep(1,length(data.mf.df$wetwteyes)),INDEX=list(data.mf.df$age.f,data.mf.df$sex), FUN=sum))

mean.pig1 <-
as.vector(tapply(data.mf.df$pigment.1,INDEX=list(data.mf.df$age.f,data.mf.df$sex), FUN=mean))
SD.pig1 <-
(as.vector(tapply(data.mf.df$pigment.1,INDEX=list(data.mf.df$age.f,data.mf.df$sex), FUN=var)))^0.5
N.pig1 <-
as.vector(tapply(rep(1,length(data.mf.df$pigment.1)),INDEX=list(data.mf.df$age.f,data.mf.df$sex), FUN=sum))
N.age <- length(levels(data.mf.df$age.f))
age.fs <- factor(rep(as.vector(levels(data.mf.df$age.f)),times=2),
levels=levels(data.mf.df$age.f))
sex.fs <- factor(rep(levels(data.mf.df$sex),each=N.age),
levels=levels(data.mf.df$sex))

mean.pig2 <-
as.vector(tapply(data.mf.df$pigment.2,INDEX=list(data.mf.df$age.f,data.mf.df$sex), FUN=mean))
SD.pig2 <-
(as.vector(tapply(data.mf.df$pigment.2,INDEX=list(data.mf.df$age.f,data.mf.df$sex), FUN=var)))^0.5
N.pig2 <-
as.vector(tapply(rep(1,length(data.mf.df$pigment.2)),INDEX=list(data.mf.df$age.f,data.mf.df$sex), FUN=sum))

mean.pig3 <-
as.vector(tapply(data.mf.df$pigment.3,INDEX=list(data.mf.df$age.f,data.mf.df$sex), FUN=mean))
SD.pig3 <-
(as.vector(tapply(data.mf.df$pigment.3,INDEX=list(data.mf.df$age.f,data.mf.df$sex), FUN=var)))^0.5
N.pig3 <-
as.vector(tapply(rep(1,length(data.mf.df$pigment.3)),INDEX=list(data.mf.df$age.f,data.mf.df$sex), FUN=sum))

CLu.pig1 <- mean.pig1+SD.pig1/(N.pig1^0.5)
CLl.pig1 <- mean.pig1-SD.pig1/(N.pig1^0.5)
CLu.pig2 <- mean.pig2+SD.pig2/(N.pig2^0.5)
CLl.pig2 <- mean.pig2-SD.pig2/(N.pig2^0.5)
CLu.pig3 <- mean.pig3+SD.pig3/(N.pig3^0.5)
CLl.pig3 <- mean.pig3-SD.pig3/(N.pig3^0.5)

CLu.wetwteyes <- mean.wetwteyes+SD.wetwteyes/(N.wetwteyes^0.5)

```

```

CLl.wetwteyes <- mean.wetwteyes-SD.wetwteyes/(N.wetwteyes^0.5)
CLu.CL <- mean.CL+SD.CL/(N.CL^0.5)
CLl.CL <- mean.CL-SD.CL/(N.CL^0.5)

age.v <- as.vector(age.fs)

Mfac <- rep(sex.fs,3)
Mx <- as.integer(rep(age.v,3))
Mdat <- c(mean.pig1,CLu.pig1,CLl.pig1)
Mgrp <- as.factor(rep(c(1:3),each=2*N.age))

Mx <- as.integer(rep(age.v,3))
Mdat <- c(mean.CL,CLu.CL,CLl.CL)
data.trel <- data.frame(Mdat,Mx,Mfac,Mgrp)
plot.trellis.sebar(data.trel,xlim=c(10,50),XLAB="Age (months)", ylab="CL
(mm)")

Mx <- as.integer(rep(age.v,3))
Mdat <- c(mean.wetwteyes,CLu.wetwteyes,CLl.wetwteyes)
data.trel <- data.frame(Mdat,Mx,Mfac,Mgrp)
plot.trellis.sebar(data.trel,xlim=c(10,50),XLAB="Age (months)",
ylab="wetwteyes")

plot(y=mean.wetwteyes, x=mean.pig1)

Mx <- as.integer(rep(age.v,3))
Mdat <- c(mean.pig1,CLu.pig1,CLl.pig1)
data.trel <- data.frame(Mdat,Mx,Mfac,Mgrp)
plot.trellis.sebar(data.trel,xlim=c(10,50),XLAB="Age (months)", ylab="Pigment
1")

Mx <- mean.wetwteyes
Mdat <- c(mean.pig1,CLu.pig1,CLl.pig1)
data.trel <- data.frame(Mdat,Mx,Mfac,Mgrp)
plot.trellis.sebar(data.trel,XLAB="Wetwteyes", ylab="Pigment 1")

Mx <- mean.wetwteyes
Mdat <- c(mean.pig3,CLu.pig3,CLl.pig3)
data.trel <- data.frame(Mdat,Mx,Mfac,Mgrp)
plot.trellis.sebar(data.trel,XLAB="Wetwteyes", ylab="Pigment 3")

plot(y=mean.wetwteyes, x=mean.pig1)

Mx <- as.integer(rep(age.v,3))
Mdat <- c(mean.pig2,CLu.pig2,CLl.pig2)
data.trel <- data.frame(Mdat,Mx,Mfac,Mgrp)
plot.trellis.sebar(data.trel,xlim=c(10,50),XLAB="Age (months)", ylab="Pigment
2")

```

```

Mx <- as.integer(rep(age.v,3))
Mdat <- c(mean.pig3,CLu.pig3,CLl.pig3)
data.trel <- data.frame(Mdat,Mx,Mfac,Mgrp)
plot.trellis.sebar(data.trel,xlim=c(10,50),XLAB="Age (months)", ylab="Pigment
3")

# analyse 36 and 48 month aged krill as logistic discriminant using pigments or CL

#data.mfa.df <- data.mf.df[data.mf.df$Age_mth>30,]

#data.mfa.df$y1 <- as.integer(data.mfa.df$Age_mth==36)
#data.mfa.df$y2 <- as.integer(data.mfa.df$Age_mth==48)

summary(data.mfa.df)

# analyse 12 and 36 month aged krill as logistic discriminant using pigments or CL

data.mfa.df <- data.mf.df[data.mf.df$Age_mth<40,]

data.mfa.df$y1 <- as.integer(data.mfa.df$Age_mth==12)
data.mfa.df$y2 <- as.integer(data.mfa.df$Age_mth==36)

summary(data.mfa.df)

glm.01 <- glm(cbind(y1,y2)~ sex+pigment.1+sex:pigment.1,
family=binomial(link=logit), data=data.mfa.df)
summary(glm.01)
glm.01 <- glm(cbind(y1,y2)~ pigment.1, family=binomial(link=logit),
data=data.mfa.df)
summary(glm.01)
glm.01 <- glm(cbind(y1,y2)~ pigment.1+ED+CL, family=binomial(link=logit),
data=data.mfa.df)
summary(glm.01)
glm.01 <- glm(cbind(y1,y2)~ pigment.1+wetwteyes+CL,
family=binomial(link=logit), data=data.mfa.df)
summary(glm.01)
glm.01 <- glm(cbind(y1,y2)~ pigment.1+wetwteyes, family=binomial(link=logit),
data=data.mfa.df)
summary(glm.01)
glm.01 <- glm(cbind(y1,y2)~ pigment.1+wetwteyes, family=binomial(link=logit),
data=data.mfa.df)
summary(glm.01)
glm.01 <- glm(cbind(y1,y2)~ pigment.3+wetwteyes, family=binomial(link=logit),
data=data.mfa.df)
summary(glm.01)
glm.01 <- glm(cbind(y1,y2)~ pigment.1+pigment.3+wetwteyes,
family=binomial(link=logit), data=data.mfa.df)
summary(glm.01)

```

```

glm.01 <- glm(cbind(y1,y2)~ sex+ pigment.1+wetwteyes + sex:pigment.1 +
sex:wetwteyes , family=binomial(link=logit), data=data.mfa.df)
summary(glm.01)

glm.01 <- glm(cbind(y1,y2)~ wetwteyes, family=binomial(link=logit),
data=data.mfa.df)
summary(glm.01)
glm.01 <- glm(cbind(y1,y2)~ pigment.1+wetwteyes+CL,
family=binomial(link=logit), data=data.mfa.df)
summary(glm.01)
glm.01 <- glm(cbind(y1,y2)~ pigment.1+wetwteyes+ED,
family=binomial(link=logit), data=data.mfa.df)
summary(glm.01)
glm.01 <- glm(cbind(y1,y2)~ pigment.1+ED, family=binomial(link=logit),
data=data.mfa.df)
summary(glm.01)

# final model 1 : pigment 1

glm.01 <- glm(cbind(y1,y2)~ pigment.1+wetwteyes, family=binomial(link=logit),
data=data.mfa.df)
summary(glm.01)

#plot(glm.01)

# include just pigment 1 in LP

glm.01 <- glm(cbind(y1,y2)~ pigment.1, family=binomial(link=logit),
data=data.mfa.df)
summary(glm.01)

glm.pred.df <-
data.frame(cbind(glm.01$fitted.values,glm.01$residuals,glm.01$y,data.mfa.df$Age
e_mth,data.mfa.df$sex,data.mfa.df$pigment.1,
data.mfa.df$wetwteyes,glm.01$linear.predictor))

names(glm.pred.df) <-
c("fv","resids","y","Age_mth","sex","pigment.1","wetwteyes","lp")

# sort glm.pred.df

glm.pred.s.df <- glm.pred.df[order(glm.pred.df$lp),]

# find optimal cutpoint on lp for %correct age 12 month + (100-(%incorrect age 12
+ %incorrect age 36 mnth))

N12 <- sum(glm.pred.s.df$y)
Ndim <- dim(glm.pred.s.df)[1]

```



```

N36 <- Ndim-N12
c(N12,N36,Ndim)
Cor12 <- rep(0,Ndim)
Cor36 <- rep(0,Ndim)

for (j in 1:Ndim) {
Cor12[j] <- sum(as.integer(glm.pred.s.df$y==1 & (seq(1,Ndim)>=j)))
Cor36[j] <- sum(as.integer(glm.pred.s.df$y==0 & (seq(1,Ndim)<j))))}

Cor12P <- 100*Cor12/N12
Cor36P <- 100*Cor36/N36
CorB <- Cor12P+Cor36P
glm.pred.s.df$Cor12P <- Cor12P
glm.pred.s.df$Cor36P <- Cor36P
glm.pred.s.df$CorB <- CorB

#edit(glm.pred.s.df)

glm.pred.opt.df <- glm.pred.s.df[order(glm.pred.s.df$CorB),]

glm.pred.opt.df[Ndim,]

# final model 2 : pigment.3

# include just pigment.3 in LP

glm.01 <- glm(cbind(y1,y2)~ sex+ pigment.3+wetwteyes + sex:pigment.3 +
sex:wetwteyes , family=binomial(link=logit), data=data.mfa.df)
summary(glm.01)

glm.01 <- glm(cbind(y1,y2)~ pigment.3, family=binomial(link=logit),
data=data.mfa.df)
summary(glm.01)

glm.01 <- glm(cbind(y1,y2)~ pigment.3+wetwteyes, family=binomial(link=logit),
data=data.mfa.df)
summary(glm.01)

#plot(glm.01)

glm.pred.df <-
data.frame(cbind(glm.01$fitted.values,glm.01$residuals,glm.01$y,data.mfa.df$Age
e_mth,data.mfa.df$sex,data.mfa.df$pigment.3,
data.mfa.df$wetwteyes,glm.01$linear.predictor))

names(glm.pred.df) <-
c("fv","resids","y","Age_mth","sex","pigment.3","wetwteyes","lp")

```

```

# sort glm.pred.df

glm.pred.s.df <- glm.pred.df[order(glm.pred.df$lp),]

# find optimal cutpoint on lp for %correct age 12 month + (100-(%incorrect age 12
+ %incorrect age 36 mnth))

N12 <- sum(glm.pred.s.df$y)
Ndim <- dim(glm.pred.s.df)[1]
N36 <- Ndim-N12
c(N12,N36,Ndim)
Cor12 <- rep(0,Ndim)
Cor36 <- rep(0,Ndim)

for (j in 1:Ndim) {
  Cor12[j] <- sum(as.integer(glm.pred.s.df$y==1 & (seq(1,Ndim)>=j)))
  Cor36[j] <- sum(as.integer(glm.pred.s.df$y==0 & (seq(1,Ndim)<=j))))}

Cor12P <- 100*Cor12/N12
Cor36P <- 100*Cor36/N36
CorB <- Cor12P+Cor36P
glm.pred.s.df$Cor12P <- Cor12P
glm.pred.s.df$Cor36P <- Cor36P
glm.pred.s.df$CorB <- CorB

#edit(glm.pred.s.df)

glm.pred.opt.df <- glm.pred.s.df[order(glm.pred.s.df$CorB),]

glm.pred.opt.df[Ndim,]

# now use carapace length instead of pigments

glm.01 <- glm(cbind(y1,y2)~ CL+wetwteyes, family=binomial(link=logit),
data=data.mfa.df)
summary(glm.01)

# use just CL

glm.01 <- glm(cbind(y1,y2)~ CL, family=binomial(link=logit), data=data.mfa.df)
summary(glm.01)

#plot(glm.01)

glm.pred.df <-
data.frame(cbind(glm.01$fitted.values,glm.01$residuals,glm.01$y,data.mfa.df$Age_
mth,data.mfa.df$sex,data.mfa.df$CL,

```

```

data.mfa.df$wetwteyes,glm.01$linear.predictor))

names(glm.pred.df) <-
c("fv","resids","y","Age_mth","sex","CL","wetwteyes","lp")

# sort glm.pred.df

glm.pred.s.df <- glm.pred.df[order(glm.pred.df$lp),]

# find optimal cutpoint on lp for %correct age 12 month + (100-(%incorrect age 12
+ %incorrect age 36 mnth))

N12 <- sum(glm.pred.s.df$y)
Ndim <- dim(glm.pred.s.df)[1]
N36 <- Ndim-N12
c(N12,N36,Ndim)
Cor12 <- rep(0,Ndim)
Cor36 <- rep(0,Ndim)

for (j in 1:Ndim) {
Cor12[j] <- sum(as.integer(glm.pred.s.df$y==1 & (seq(1,Ndim)>=j)))
Cor36[j] <- sum(as.integer(glm.pred.s.df$y==0 & (seq(1,Ndim)<j)))}

Cor12P <- 100*Cor12/N12
Cor36P <- 100*Cor36/N36
CorB <- Cor12P+Cor36P
glm.pred.s.df$Cor12P <- Cor12P
glm.pred.s.df$Cor36P <- Cor36P
glm.pred.s.df$CorB <- CorB

#edit(glm.pred.s.df)

glm.pred.opt.df <- glm.pred.s.df[order(glm.pred.s.df$CorB),]

glm.pred.opt.df[Ndim,]

glm.01 <- glm(cbind(y1,y2)~ sex+CL+sex:CL, family=binomial(link=logit),
data=data.mfa.df)
summary(glm.01)

glm.01 <- glm(cbind(y1,y2)~ sex+pigment.1+sex:pigment.1+CL+sex:CL,
family=binomial(link=logit), data=data.mfa.df)
summary(glm.01)

"plot.trellis.sebar"<-
function(data.df,xlim,ylim,XLAB,ylab)
{

```

```

background <- trellis.par.get("background")
background$col <- "white"
trellis.par.set("background",background)
print(xyplot(Mdat ~ Mx | Mfac, data = data.df, groups=Mgrp,
  panel = function(x,y,subscripts,groups) {
    panel.xyplot(x = x[groups[subscripts]==1] , y = y[groups[subscripts]==1],
      type="p",cex=1.3, col=2)
    lsegments(x1=x[groups[subscripts]==2], y1=y[groups[subscripts]==2],
      x2=x[groups[subscripts]==3], y2=y[groups[subscripts]==3]}),
  xlab = XLAB, ylab = ylab, ylim=ylim, xlim=xlim,layout=c(2,1),
  cex=1.3))
}

```

### Output

R version 2.7.0 (2008-04-22)  
 Copyright (C) 2008 The R Foundation for Statistical Computing  
 ISBN 3-900051-07-0

R is free software and comes with ABSOLUTELY NO WARRANTY.  
 You are welcome to redistribute it under certain conditions.  
 Type 'license()' or 'licence()' for distribution details.

Natural language support but running in an English locale

R is a collaborative project with many contributors.  
 Type 'contributors()' for more information and  
 'citation()' on how to cite R or R packages in publications.

Type 'demo()' for some demos, 'help()' for on-line help, or  
 'help.start()' for an HTML browser interface to help.  
 Type 'q()' to quit R.

```

> 29000/26
[1] 1115.385
> # Angela's pigment analyses
> # part 1 Cumulative pigment vs age
>
> library(lattice)
>
> data.df <- read.csv(file="Part1.csv")
>
> summary(data.df)

```

|                                      | Group | Age_mth       | CL             |
|--------------------------------------|-------|---------------|----------------|
| Known age - 1 month (0.08 year old): | 2     | Min. : 1.00   | Min. : 2.000   |
| Known age - 1 year old               | :25   | 1st Qu.:12.00 | 1st Qu.: 9.792 |
| Known age - 3 year old               | :12   | Median :12.00 | Median :11.179 |
| Known age - 4 year old               | :12   | Mean :25.69   | Mean :10.916   |
|                                      |       | 3rd Qu.:36.00 | 3rd Qu.:12.536 |

Max. :48.00 Max. :15.299

TL ED wetwteyes sex FAP\_Ex280Em625  
Mode:logical Min. :1.484 Min. :0.004400 f:37 Min. :0.900  
NA's:51 1st Qu.:1.704 1st Qu.:0.006400 j:2 1st Qu.:1.050  
Median :1.858 Median :0.007700 m:12 Median :1.300  
Mean :1.901 Mean :0.008798 Mean :1.347  
3rd Qu.:2.131 3rd Qu.:0.011850 3rd Qu.:1.600  
Max. :2.316 Max. :0.015100 Max. :2.000  
NA's :2.000

FAP\_Ex355Em510 FAP\_Ex463Em620 protein pigment.1  
Min. :0.1000 Min. :0.1000 Min. :1.000 Min. :0.2944  
1st Qu.:0.2000 1st Qu.:0.1000 1st Qu.:1.550 1st Qu.:0.5598  
Median :0.2000 Median :0.2000 Median :1.800 Median :0.7210  
Mean :0.2392 Mean :0.1765 Mean :1.863 Mean :0.7635  
3rd Qu.:0.2500 3rd Qu.:0.2000 3rd Qu.:2.100 3rd Qu.:0.9447  
Max. :0.8000 Max. :0.4000 Max. :3.500 Max. :1.2587

pigment.2 pigment.3  
Min. :0.05129 Min. :0.03224  
1st Qu.:0.08309 1st Qu.:0.07150  
Median :0.10178 Median :0.10368  
Mean :0.13587 Mean :0.10298  
3rd Qu.:0.15232 3rd Qu.:0.12988  
Max. :0.46182 Max. :0.22786

```
>
> data.mf.df <- data.df[data.df$sex!="j",]
>
> data.mf.df$sex <- as.factor(as.character(data.mf.df$sex))
>
> xyplot(pigment.3 ~ Age_mth | sex, data=data.mf.df, type="p")
>
> xyplot(CL ~ Age_mth | sex, data=data.mf.df, type="p")
>
> xyplot(CL ~ pigment.3 | sex, data=data.mf.df, type="p")
>
> xyplot( pigment.1 ~ wetwteyes | sex, data=data.mf.df, type="p")
>
> xyplot( pigment.3 ~ wetwteyes | sex, data=data.mf.df, type="p")
>
> xyplot(wetwteyes ~ pigment.3 | sex, data=data.mf.df, type="p")
>
> xyplot(wetwteyes ~ Age_mth | sex, data=data.mf.df, type="p")
>
>
> data.mf.df
```

|   | Group                  | Age_mth | CL        | TL | ED       | wetwteyes | sex |
|---|------------------------|---------|-----------|----|----------|-----------|-----|
| 3 | Known age - 1 year old | 12      | 11.288073 | NA | 1.595922 | 0.0058    | f   |
| 4 | Known age - 1 year old | 12      | 11.700210 | NA | 1.725335 | 0.0066    | f   |

|    |                        |    |           |    |          |        |   |
|----|------------------------|----|-----------|----|----------|--------|---|
| 5  | Known age - 1 year old | 12 | 8.363820  | NA | 1.484178 | 0.0044 | f |
| 6  | Known age - 1 year old | 12 | 11.661401 | NA | 1.727280 | 0.0069 | f |
| 7  | Known age - 1 year old | 12 | 10.263890 | NA | 1.631814 | 0.0064 | f |
| 8  | Known age - 1 year old | 12 | 8.413057  | NA | 1.576340 | 0.0045 | f |
| 9  | Known age - 1 year old | 12 | 8.891858  | NA | 1.657336 | 0.0092 | f |
| 10 | Known age - 1 year old | 12 | 11.179494 | NA | 1.648194 | 0.0055 | f |
| 11 | Known age - 1 year old | 12 | 10.949222 | NA | 1.722900 | 0.0064 | f |
| 12 | Known age - 1 year old | 12 | 10.568770 | NA | 1.684466 | 0.0052 | f |
| 13 | Known age - 1 year old | 12 | 10.957500 | NA | 1.795902 | 0.0063 | f |
| 14 | Known age - 1 year old | 12 | 11.327198 | NA | 1.673968 | 0.0065 | f |
| 15 | Known age - 1 year old | 12 | 11.381613 | NA | 1.635924 | 0.0137 | f |
| 16 | Known age - 1 year old | 12 | 10.467970 | NA | 1.649721 | 0.0063 | f |
| 17 | Known age - 1 year old | 12 | 10.715805 | NA | 1.738422 | 0.0070 | f |
| 18 | Known age - 1 year old | 12 | 10.598277 | NA | 1.593817 | 0.0050 | f |
| 19 | Known age - 1 year old | 12 | 9.864768  | NA | 1.643603 | 0.0054 | f |
| 20 | Known age - 1 year old | 12 | 9.290488  | NA | 1.801503 | 0.0056 | m |
| 21 | Known age - 1 year old | 12 | 9.049633  | NA | 1.704284 | 0.0056 | m |
| 22 | Known age - 1 year old | 12 | 9.481530  | NA | 1.814503 | 0.0069 | m |
| 23 | Known age - 1 year old | 12 | 10.320496 | NA | 1.970212 | 0.0070 | m |
| 24 | Known age - 1 year old | 12 | 9.100694  | NA | 1.766208 | 0.0069 | m |
| 25 | Known age - 1 year old | 12 | 9.842874  | NA | 1.705269 | 0.0068 | m |
| 26 | Known age - 1 year old | 12 | 9.497807  | NA | 1.858387 | 0.0072 | m |
| 27 | Known age - 1 year old | 12 | 9.718938  | NA | 1.760018 | 0.0077 | m |
| 28 | Known age - 3 year old | 36 | 11.359169 | NA | 1.888854 | 0.0048 | f |
| 29 | Known age - 3 year old | 36 | 12.747440 | NA | 2.007775 | 0.0098 | f |
| 30 | Known age - 3 year old | 36 | 11.765606 | NA | 1.888854 | 0.0087 | f |
| 31 | Known age - 3 year old | 36 | 11.052698 | NA | 1.879944 | 0.0077 | f |
| 32 | Known age - 3 year old | 36 | 14.373876 | NA | 2.293502 | 0.0151 | f |
| 33 | Known age - 3 year old | 36 | 14.249524 | NA | 2.236049 | 0.0136 | f |
| 34 | Known age - 3 year old | 36 | 14.274187 | NA | 2.106928 | 0.0118 | f |
| 35 | Known age - 3 year old | 36 | 14.085510 | NA | 2.178237 | 0.0126 | f |
| 36 | Known age - 3 year old | 36 | 13.048656 | NA | 2.191684 | 0.0121 | f |
| 37 | Known age - 3 year old | 36 | 15.298827 | NA | 2.213029 | 0.0134 | f |
| 38 | Known age - 3 year old | 36 | 9.741456  | NA | 2.181318 | 0.0110 | m |
| 39 | Known age - 3 year old | 36 | 12.859950 | NA | 2.222115 | 0.0142 | m |
| 40 | Known age - 4 year old | 48 | 11.474171 | NA | 2.048750 | 0.0119 | f |
| 41 | Known age - 4 year old | 48 | 12.744872 | NA | 2.266995 | 0.0146 | f |
| 42 | Known age - 4 year old | 48 | 13.140907 | NA | 2.315722 | 0.0140 | f |
| 43 | Known age - 4 year old | 48 | 12.916487 | NA | 2.009447 | 0.0119 | f |
| 44 | Known age - 4 year old | 48 | 11.319784 | NA | 1.967227 | 0.0077 | f |
| 45 | Known age - 4 year old | 48 | 13.100727 | NA | 2.131094 | 0.0095 | f |
| 46 | Known age - 4 year old | 48 | 12.647410 | NA | 2.209993 | 0.0107 | f |
| 47 | Known age - 4 year old | 48 | 10.860308 | NA | 1.932787 | 0.0096 | f |
| 48 | Known age - 4 year old | 48 | 11.546301 | NA | 2.116865 | 0.0123 | f |
| 49 | Known age - 4 year old | 48 | 12.423806 | NA | 2.196275 | 0.0127 | f |
| 50 | Known age - 4 year old | 48 | 9.412485  | NA | 1.843875 | 0.0083 | m |
| 51 | Known age - 4 year old | 48 | 11.362568 | NA | 2.255115 | 0.0095 | m |

FAP\_Ex280Em625 FAP\_Ex355Em510 FAP\_Ex463Em620 protein pigment.1  
pigment.2

3 1.2 0.3 0.1 1.9 0.6588995 0.14226618

|    |     |     |     |     |           |            |
|----|-----|-----|-----|-----|-----------|------------|
| 4  | 1.2 | 0.3 | 0.2 | 2.0 | 0.5891918 | 0.14891308 |
| 5  | 1.1 | 0.2 | 0.1 | 1.6 | 0.6626846 | 0.15572129 |
| 6  | 1.0 | 0.2 | 0.1 | 1.7 | 0.5488033 | 0.10178093 |
| 7  | 1.2 | 0.2 | 0.2 | 1.8 | 0.6714291 | 0.09930244 |
| 8  | 1.0 | 0.2 | 0.1 | 1.1 | 0.8975459 | 0.20407591 |
| 9  | 1.3 | 0.3 | 0.2 | 1.5 | 0.8366448 | 0.19480930 |
| 10 | 1.0 | 0.2 | 0.1 | 1.6 | 0.6654294 | 0.10095767 |
| 11 | 1.1 | 0.2 | 0.1 | 2.2 | 0.4771378 | 0.07419779 |
| 12 | 1.0 | 0.2 | 0.1 | 1.9 | 0.5491546 | 0.10886623 |
| 13 | 1.0 | 0.2 | 0.1 | 1.5 | 0.6422108 | 0.11148521 |
| 14 | 1.3 | 0.2 | 0.1 | 1.8 | 0.7044182 | 0.11905361 |
| 15 | 1.0 | 0.3 | 0.1 | 1.6 | 0.6514730 | 0.17103931 |
| 16 | 0.9 | 0.2 | 0.1 | 3.1 | 0.2943543 | 0.06489968 |
| 17 | 1.0 | 0.2 | 0.1 | 2.3 | 0.4402388 | 0.08309628 |
| 18 | 1.0 | 0.2 | 0.1 | 1.7 | 0.5704283 | 0.12598513 |
| 19 | 0.9 | 0.1 | 0.1 | 1.3 | 0.6923389 | 0.09407588 |
| 20 | 1.3 | 0.2 | 0.1 | 2.4 | 0.5210804 | 0.08913645 |
| 21 | 0.9 | 0.1 | 0.1 | 2.0 | 0.4621673 | 0.05996865 |
| 22 | 1.1 | 0.1 | 0.1 | 2.0 | 0.5480637 | 0.07526369 |
| 23 | 1.1 | 0.2 | 0.1 | 2.1 | 0.5374444 | 0.09432079 |
| 24 | 1.1 | 0.2 | 0.1 | 3.5 | 0.3151426 | 0.06047176 |
| 25 | 1.1 | 0.2 | 0.1 | 2.1 | 0.5403241 | 0.08388574 |
| 26 | 1.0 | 0.1 | 0.1 | 2.0 | 0.4910010 | 0.06825508 |
| 27 | 1.3 | 0.1 | 0.2 | 2.4 | 0.5454530 | 0.06010937 |
| 28 | 1.0 | 0.1 | 0.2 | 1.0 | 0.9819990 | 0.13132635 |
| 29 | 1.5 | 0.2 | 0.2 | 1.2 | 1.2587051 | 0.18073336 |
| 30 | 1.2 | 0.3 | 0.2 | 1.5 | 0.8121182 | 0.16748461 |
| 31 | 1.6 | 0.6 | 0.2 | 1.3 | 1.2237513 | 0.46181738 |
| 32 | 1.6 | 0.2 | 0.2 | 2.4 | 0.6586262 | 0.08791442 |
| 33 | 1.7 | 0.3 | 0.3 | 2.3 | 0.7612819 | 0.11883478 |
| 34 | 1.5 | 0.1 | 0.2 | 1.8 | 0.8483388 | 0.07992250 |
| 35 | 1.7 | 0.4 | 0.4 | 1.9 | 0.9024364 | 0.19311909 |
| 36 | 1.4 | 0.5 | 0.3 | 1.7 | 0.7908683 | 0.30871266 |
| 37 | 1.4 | 0.3 | 0.3 | 2.4 | 0.5924829 | 0.12611867 |
| 38 | 1.6 | 0.2 | 0.2 | 1.3 | 1.2190976 | 0.16305773 |
| 39 | 2.0 | 0.8 | 0.3 | 2.1 | 0.9272111 | 0.36871460 |
| 40 | 1.4 | 0.6 | 0.2 | 1.4 | 0.9430843 | 0.42172994 |
| 41 | 2.0 | 0.2 | 0.3 | 2.5 | 0.8123771 | 0.09759920 |
| 42 | 1.6 | 0.2 | 0.2 | 2.1 | 0.7729030 | 0.08308886 |
| 43 | 1.6 | 0.2 | 0.2 | 1.6 | 1.0597460 | 0.11266976 |
| 44 | 1.7 | 0.2 | 0.2 | 1.5 | 1.1154632 | 0.12468288 |
| 45 | 1.6 | 0.5 | 0.2 | 1.6 | 0.9561093 | 0.31555933 |
| 46 | 1.9 | 0.2 | 0.2 | 1.9 | 0.9694959 | 0.09626772 |
| 47 | 1.6 | 0.2 | 0.2 | 1.5 | 1.0545982 | 0.12269084 |
| 48 | 1.7 | 0.2 | 0.2 | 2.1 | 0.8311251 | 0.07258036 |
| 49 | 1.7 | 0.2 | 0.2 | 2.4 | 0.7210169 | 0.08165462 |
| 50 | 1.9 | 0.2 | 0.3 | 2.0 | 0.9462808 | 0.10137470 |
| 51 | 1.8 | 0.2 | 0.2 | 1.7 | 1.0632125 | 0.10040351 |

pigment.3  
3 0.07665098

```

4 0.07607523
5 0.08596314
6 0.05973428
7 0.08671285
8 0.10620236
9 0.10540777
10 0.06950616
11 0.05332607
12 0.07279933
13 0.07950338
14 0.08197814
15 0.07036922
16 0.03223858
17 0.04715741
18 0.06489329
19 0.08714236
20 0.04721561
21 0.05302330
22 0.07262235
23 0.04653295
24 0.03751859
25 0.06698061
26 0.05578276
27 0.07650611
28 0.14758920
29 0.16758736
30 0.10657435
31 0.12510161
32 0.08413845
33 0.11098638
34 0.10872911
35 0.22785549
36 0.19284580
37 0.13565794
38 0.14814191
39 0.12489784
40 0.12381314
41 0.12032335
42 0.11058145
43 0.13835599
44 0.13578645
45 0.12762292
46 0.11565794
47 0.13213005
48 0.09964927
49 0.10368307
50 0.14090993
51 0.14793070
> data.mf.df$age.f <- as.factor(data.mf.df$Age_mth)
>

```



```

> mean.CL <-
as.vector(tapply(data.mf.df$CL,INDEX=list(data.mf.df$age.f,data.mf.df$sex),
FUN=mean))
> SD.CL <-
(as.vector(tapply(data.mf.df$CL,INDEX=list(data.mf.df$age.f,data.mf.df$sex),
FUN=var)))^0.5
> N.CL <-
as.vector(tapply(rep(1,length(data.mf.df$CL)),INDEX=list(data.mf.df$age.f,data.
mf.df$sex), FUN=sum))
>
> mean.wetwteyes <-
as.vector(tapply(data.mf.df$wetwteyes,INDEX=list(data.mf.df$age.f,data.mf.df$sex),
FUN=mean))
> SD.wetwteyes <-
(as.vector(tapply(data.mf.df$wetwteyes,INDEX=list(data.mf.df$age.f,data.mf.df$sex),
FUN=var)))^0.5
> N.wetwteyes <-
as.vector(tapply(rep(1,length(data.mf.df$wetwteyes)),INDEX=list(data.mf.df$age.
f,data.mf.df$sex), FUN=sum))
>
> mean.pig1 <-
as.vector(tapply(data.mf.df$pigment.1,INDEX=list(data.mf.df$age.f,data.mf.df$sex),
FUN=mean))
> SD.pig1 <-
(as.vector(tapply(data.mf.df$pigment.1,INDEX=list(data.mf.df$age.f,data.mf.df$sex),
FUN=var)))^0.5
> N.pig1 <-
as.vector(tapply(rep(1,length(data.mf.df$pigment.1)),INDEX=list(data.mf.df$age.f
,data.mf.df$sex), FUN=sum))
> N.age <- length(levels(data.mf.df$age.f))
> age.fs <- factor(rep(as.vector(levels(data.mf.df$age.f)),times=2),
levels=levels(data.mf.df$age.f))
> sex.fs <- factor(rep(levels(data.mf.df$sex),each=N.age),
levels=levels(data.mf.df$sex))
>
> mean.pig2 <-
as.vector(tapply(data.mf.df$pigment.2,INDEX=list(data.mf.df$age.f,data.mf.df$sex),
FUN=mean))
> SD.pig2 <-
(as.vector(tapply(data.mf.df$pigment.2,INDEX=list(data.mf.df$age.f,data.mf.df$sex),
FUN=var)))^0.5
> N.pig2 <-
as.vector(tapply(rep(1,length(data.mf.df$pigment.2)),INDEX=list(data.mf.df$age.f
,data.mf.df$sex), FUN=sum))
>
> mean.pig3 <-
as.vector(tapply(data.mf.df$pigment.3,INDEX=list(data.mf.df$age.f,data.mf.df$sex),
FUN=mean))

```

```

> SD.pig3 <-
(as.vector(tapply(data.mf.df$pigment.3,INDEX=list(data.mf.df$age.f,data.mf.df$sex), FUN=var)))^0.5
> N.pig3 <-
as.vector(tapply(rep(1,length(data.mf.df$pigment.3)),INDEX=list(data.mf.df$age.f,data.mf.df$sex), FUN=sum))
>
> CLu.pig1 <- mean.pig1+SD.pig1/(N.pig1^0.5)
> CLl.pig1 <- mean.pig1-SD.pig1/(N.pig1^0.5)
> CLu.pig2 <- mean.pig2+SD.pig2/(N.pig2^0.5)
> CLl.pig2 <- mean.pig2-SD.pig2/(N.pig2^0.5)
> CLu.pig3 <- mean.pig3+SD.pig3/(N.pig3^0.5)
> CLl.pig3 <- mean.pig3-SD.pig3/(N.pig3^0.5)
>
> CLu.wetwteyes <- mean.wetwteyes+SD.wetwteyes/(N.wetwteyes^0.5)
> CLl.wetwteyes <- mean.wetwteyes-SD.wetwteyes/(N.wetwteyes^0.5)
> CLu.CL <- mean.CL+SD.CL/(N.CL^0.5)
> CLl.CL <- mean.CL-SD.CL/(N.CL^0.5)
>
>
> "plot.trellis.sebar"<-
+ function(data.df,xlim,ylim,XLAB,ylab)
+ {
+   background <- trellis.par.get("background")
+   background$col <- "white"
+   trellis.par.set("background",background)
+   print(xyplot(Mdat ~ Mx | Mfac, data = data.df, groups=Mgrp,
+     panel = function(x,y,subscripts,groups) {
+       panel.xyplot(x = x[groups[subscripts]==1], y =
y[groups[subscripts]==1],
+       type="p",cex=1.3, col=2)
+       lsegments(x1=x[groups[subscripts]==2], y1=y[groups[subscripts]==2],
+       x2=x[groups[subscripts]==3], y2=y[groups[subscripts]==3]}),
+     xlab = XLAB, ylab = ylab, ylim=ylim, xlim=xlim,layout=c(2,1),
+     cex=1.3))
+ }
> age.v <- as.vector(age.fs)
>
> Mfac <- rep(sex.fs,3)
> Mx <- as.integer(rep(age.v,3))
> Mdat <- c(mean.pig1,CLu.pig1,CLl.pig1)
> Mgrp <- as.factor(rep(c(1:3),each=2*N.age))
>
> Mx <- as.integer(rep(age.v,3))
> Mdat <- c(mean.CL,CLu.CL,CLl.CL)
> data.trel <- data.frame(Mdat,Mx,Mfac,Mgrp)
> plot.trellis.sebar(data.trel,xlim=c(10,50),XLAB="Age (months)", ylab="CL
(mm)")
>
> Mx <- as.integer(rep(age.v,3))

```

```

> Mdat <- c(mean.wetwteyes, CLu.wetwteyes, CLl.wetwteyes)
> data.trel <- data.frame(Mdat, Mx, Mfac, Mgrp)
> plot.trellis.sebar(data.trel, xlim=c(10,50), XLAB="Age (months)",
ylab="wetwteyes")
>
> plot(y=mean.wetwteyes, x=mean.pig1)
>
> Mx <- as.integer(rep(age.v,3))
> Mdat <- c(mean.pig1, CLu.pig1, CLl.pig1)
> data.trel <- data.frame(Mdat, Mx, Mfac, Mgrp)
> plot.trellis.sebar(data.trel, xlim=c(10,50), XLAB="Age (months)", ylab="Pigment
1")
>
> Mx <- mean.wetwteyes
> Mdat <- c(mean.pig1, CLu.pig1, CLl.pig1)
> data.trel <- data.frame(Mdat, Mx, Mfac, Mgrp)
> plot.trellis.sebar(data.trel, XLAB="Wetwteyes", ylab="Pigment 1")
>
> Mx <- mean.wetwteyes
> Mdat <- c(mean.pig3, CLu.pig3, CLl.pig3)
> data.trel <- data.frame(Mdat, Mx, Mfac, Mgrp)
> plot.trellis.sebar(data.trel, XLAB="Wetwteyes", ylab="Pigment 3")
>
>
> plot(y=mean.wetwteyes, x=mean.pig1)
>
> Mx <- as.integer(rep(age.v,3))
> Mdat <- c(mean.pig2, CLu.pig2, CLl.pig2)
> data.trel <- data.frame(Mdat, Mx, Mfac, Mgrp)
> plot.trellis.sebar(data.trel, xlim=c(10,50), XLAB="Age (months)", ylab="Pigment
2")
>
> Mx <- as.integer(rep(age.v,3))
> Mdat <- c(mean.pig3, CLu.pig3, CLl.pig3)
> data.trel <- data.frame(Mdat, Mx, Mfac, Mgrp)
> plot.trellis.sebar(data.trel, xlim=c(10,50), XLAB="Age (months)", ylab="Pigment
3")
>
>
> # analyse 12 and 36 month aged krill as logistic discriminant using pigments or
CL
>
> data.mfa.df <- data.mf.df[data.mf.df$Age_mth<40,]
>
> data.mfa.df$y1 <- as.integer(data.mfa.df$Age_mth==12)
> data.mfa.df$y2 <- as.integer(data.mfa.df$Age_mth==36)
>
> summary(data.mfa.df)
      Group   Age_mth      CL
Known age - 1 month (0.08 year old): 0   Min.  :12.00   Min.  : 8.364

```

```

Known age - 1 year old      :25 1st Qu.:12.00 1st Qu.: 9.741
Known age - 3 year old      :12 Median :12.00 Median :10.949
Known age - 4 year old      :0 Mean :19.78 Mean :11.074
                             3rd Qu.:36.00 3rd Qu.:11.700
                             Max. :36.00 Max. :15.299

TL      ED      wetwteyes  sex  FAP_Ex280Em625
Mode:logical Min. :1.484 Min. :0.004400 f:27 Min. :0.900
NA's:37 1st Qu.:1.657 1st Qu.:0.005800 m:10 1st Qu.:1.000
      Median :1.760 Median :0.006900 Median :1.100
      Mean :1.834 Mean :0.008097 Mean :1.224
      3rd Qu.:1.970 3rd Qu.:0.009800 3rd Qu.:1.400
      Max. :2.294 Max. :0.015100 Max. :2.000
FAP_Ex355Em510 FAP_Ex463Em620 protein pigment.1
Min. :0.1000 Min. :0.1000 Min. :1.000 Min. :0.2944
1st Qu.:0.2000 1st Qu.:0.1000 1st Qu.:1.600 1st Qu.:0.5455
Median :0.2000 Median :0.1000 Median :1.900 Median :0.6586
Mean :0.2405 Mean :0.1595 Mean :1.892 Mean :0.6889
3rd Qu.:0.3000 3rd Qu.:0.2000 3rd Qu.:2.100 3rd Qu.:0.8121
Max. :0.8000 Max. :0.4000 Max. :3.500 Max. :1.2587
pigment.2 pigment.3 age.f y1 y2
Min. :0.05997 Min. :0.03224 12:25 Min. :0.0000 Min. :0.0000
1st Qu.:0.08389 1st Qu.:0.06489 36:12 1st Qu.:0.0000 1st Qu.:0.0000
Median :0.11149 Median :0.07950 48:0 Median :1.0000 Median :0.0000
Mean :0.13729 Mean :0.09167 Mean :0.6757 Mean :0.3243
3rd Qu.:0.16306 3rd Qu.:0.10873 3rd Qu.:1.0000 3rd Qu.:1.0000
Max. :0.46182 Max. :0.22786 Max. :1.0000 Max. :1.0000
>
> glm.01 <- glm(cbind(y1,y2)~ sex+pigment.1+sex:pigment.1,
family=binomial(link=logit), data=data.mfa.df)
Warning message:
In glm.fit(x = X, y = Y, weights = weights, start = start, etastart = etastart, :
fitted probabilities numerically 0 or 1 occurred
> summary(glm.01)

Call:
glm(formula = cbind(y1, y2) ~ sex + pigment.1 + sex:pigment.1,
family = binomial(link = logit), data = data.mfa.df)

Deviance Residuals:
    Min       1Q   Median       3Q      Max
-2.0635159 -0.1086555  0.0001488  0.6433320  1.7390905

Coefficients:
            Estimate Std. Error z value Pr(>|z|)
(Intercept)    8.345     3.280  2.544  0.0110 *
sexm           61.508    9673.995  0.006  0.9949
pigment.1     -10.705     4.443 -2.409  0.0160 *
sexm:pigment.1 -83.774   13879.595 -0.006  0.9952
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

```

(Dispersion parameter for binomial family taken to be 1)

Null deviance: 46.626 on 36 degrees of freedom  
Residual deviance: 23.112 on 33 degrees of freedom  
AIC: 31.112

Number of Fisher Scoring iterations: 18

```
> glm.01 <- glm(cbind(y1,y2)~ pigment.1, family=binomial(link=logit),  
data=data.mfa.df)  
> summary(glm.01)
```

Call:

```
glm(formula = cbind(y1, y2) ~ pigment.1, family = binomial(link = logit),  
data = data.mfa.df)
```

Deviance Residuals:

| Min      | 1Q       | Median  | 3Q      | Max     |
|----------|----------|---------|---------|---------|
| -2.19432 | -0.08578 | 0.28057 | 0.58332 | 1.86279 |

Coefficients:

|             | Estimate | Std. Error | z value | Pr(> z )   |
|-------------|----------|------------|---------|------------|
| (Intercept) | 9.798    | 3.116      | 3.144   | 0.00167 ** |
| pigment.1   | -12.634  | 4.248      | -2.974  | 0.00294 ** |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

(Dispersion parameter for binomial family taken to be 1)

Null deviance: 46.626 on 36 degrees of freedom  
Residual deviance: 24.162 on 35 degrees of freedom  
AIC: 28.162

Number of Fisher Scoring iterations: 6

```
> glm.01 <- glm(cbind(y1,y2)~ pigment.1+ED+CL, family=binomial(link=logit),  
data=data.mfa.df)
```

Warning messages:

1: In glm.fit(x = X, y = Y, weights = weights, start = start, etastart = etastart, :  
algorithm did not converge

2: In glm.fit(x = X, y = Y, weights = weights, start = start, etastart = etastart, :  
fitted probabilities numerically 0 or 1 occurred

```
> summary(glm.01)
```

Call:

```
glm(formula = cbind(y1, y2) ~ pigment.1 + ED + CL, family = binomial(link =  
logit),  
data = data.mfa.df)
```

Deviance Residuals:

| Min        | 1Q         | Median    | 3Q        | Max       |
|------------|------------|-----------|-----------|-----------|
| -2.130e-05 | -2.107e-08 | 2.107e-08 | 2.107e-08 | 1.765e-05 |

Coefficients:

|             | Estimate | Std. Error | z value   | Pr(> z ) |
|-------------|----------|------------|-----------|----------|
| (Intercept) | 422.84   | 592940.63  | 0.001     | 1        |
| pigment.1   | -161.19  | 346543.42  | -0.000465 | 1        |
| ED          | -113.49  | 370952.25  | -0.000306 | 1        |
| CL          | -8.48    | 49848.21   | -0.000170 | 1        |

(Dispersion parameter for binomial family taken to be 1)

Null deviance: 4.6626e+01 on 36 degrees of freedom  
Residual deviance: 9.9697e-10 on 33 degrees of freedom  
AIC: 8

Number of Fisher Scoring iterations: 25

```
> glm.01 <- glm(cbind(y1,y2)~ pigment.1+wetwteyes+CL,  
family=binomial(link=logit), data=data.mfa.df)
```

Warning messages:

```
1: In glm.fit(x = X, y = Y, weights = weights, start = start, etastart = etastart, :  
algorithm did not converge  
2: In glm.fit(x = X, y = Y, weights = weights, start = start, etastart = etastart, :  
fitted probabilities numerically 0 or 1 occurred  
> summary(glm.01)
```

Call:

```
glm(formula = cbind(y1, y2) ~ pigment.1 + wetwteyes + CL, family =  
binomial(link = logit),  
data = data.mfa.df)
```

Deviance Residuals:

| Min        | 1Q         | Median    | 3Q        | Max       |
|------------|------------|-----------|-----------|-----------|
| -2.883e-05 | -2.107e-08 | 2.107e-08 | 2.107e-08 | 2.827e-05 |

Coefficients:

|             | Estimate   | Std. Error | z value   | Pr(> z ) |
|-------------|------------|------------|-----------|----------|
| (Intercept) | 4.518e+02  | 4.803e+05  | 0.001     | 1        |
| pigment.1   | -2.885e+02 | 3.227e+05  | -0.001    | 1        |
| wetwteyes   | -1.738e+03 | 1.049e+07  | -0.000166 | 1        |
| CL          | -1.904e+01 | 3.827e+04  | -0.000497 | 1        |

(Dispersion parameter for binomial family taken to be 1)

Null deviance: 4.6626e+01 on 36 degrees of freedom  
Residual deviance: 1.8272e-09 on 33 degrees of freedom  
AIC: 8

Number of Fisher Scoring iterations: 25

```
> glm.01 <- glm(cbind(y1,y2)~ pigment.1+wetwteyes,  
family=binomial(link=logit), data=data.mfa.df)  
> summary(glm.01)
```

Call:

```
glm(formula = cbind(y1, y2) ~ pigment.1 + wetwteyes, family = binomial(link =  
logit),  
data = data.mfa.df)
```

Deviance Residuals:

| Min      | 1Q       | Median  | 3Q      | Max     |
|----------|----------|---------|---------|---------|
| -1.39339 | -0.03415 | 0.03133 | 0.07907 | 1.64272 |

Coefficients:

|             | Estimate | Std. Error | z value | Pr(> z ) |
|-------------|----------|------------|---------|----------|
| (Intercept) | 22.31    | 11.03      | 2.023   | 0.0431 * |
| pigment.1   | -19.50   | 10.22      | -1.907  | 0.0565 . |
| wetwteyes   | -765.84  | 362.27     | -2.114  | 0.0345 * |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

(Dispersion parameter for binomial family taken to be 1)

Null deviance: 46.626 on 36 degrees of freedom  
Residual deviance: 10.481 on 34 degrees of freedom  
AIC: 16.481

Number of Fisher Scoring iterations: 8

```
> glm.01 <- glm(cbind(y1,y2)~ pigment.1+wetwteyes,  
family=binomial(link=logit), data=data.mfa.df)  
> summary(glm.01)
```

Call:

```
glm(formula = cbind(y1, y2) ~ pigment.1 + wetwteyes, family = binomial(link =  
logit),  
data = data.mfa.df)
```

Deviance Residuals:

| Min      | 1Q       | Median  | 3Q      | Max     |
|----------|----------|---------|---------|---------|
| -1.39339 | -0.03415 | 0.03133 | 0.07907 | 1.64272 |

Coefficients:

|             | Estimate | Std. Error | z value | Pr(> z ) |
|-------------|----------|------------|---------|----------|
| (Intercept) | 22.31    | 11.03      | 2.023   | 0.0431 * |
| pigment.1   | -19.50   | 10.22      | -1.907  | 0.0565 . |
| wetwteyes   | -765.84  | 362.27     | -2.114  | 0.0345 * |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

(Dispersion parameter for binomial family taken to be 1)

Null deviance: 46.626 on 36 degrees of freedom  
Residual deviance: 10.481 on 34 degrees of freedom  
AIC: 16.481

Number of Fisher Scoring iterations: 8

```
> glm.01 <- glm(cbind(y1,y2)~ pigment.3+wetwteyes,  
family=binomial(link=logit), data=data.mfa.df)  
Warning message:  
In glm.fit(x = X, y = Y, weights = weights, start = start, etastart = etastart, :  
fitted probabilities numerically 0 or 1 occurred  
> summary(glm.01)
```

Call:

```
glm(formula = cbind(y1, y2) ~ pigment.3 + wetwteyes, family = binomial(link =  
logit),  
data = data.mfa.df)
```

Deviance Residuals:

| Min        | 1Q         | Median    | 3Q        | Max       |
|------------|------------|-----------|-----------|-----------|
| -1.228e+00 | -8.136e-06 | 1.047e-05 | 7.116e-04 | 1.321e+00 |

Coefficients:

|             | Estimate | Std. Error | z value | Pr(> z ) |
|-------------|----------|------------|---------|----------|
| (Intercept) | 54.35    | 61.23      | 0.888   | 0.375    |
| pigment.3   | -365.87  | 429.02     | -0.853  | 0.394    |
| wetwteyes   | -1751.25 | 1849.92    | -0.947  | 0.344    |

(Dispersion parameter for binomial family taken to be 1)

Null deviance: 46.626 on 36 degrees of freedom  
Residual deviance: 3.402 on 34 degrees of freedom  
AIC: 9.402

Number of Fisher Scoring iterations: 12

```
> glm.01 <- glm(cbind(y1,y2)~ pigment.1+pigment.3+wetwteyes,  
family=binomial(link=logit), data=data.mfa.df)  
Warning messages:  
1: In glm.fit(x = X, y = Y, weights = weights, start = start, etastart = etastart, :  
algorithm did not converge  
2: In glm.fit(x = X, y = Y, weights = weights, start = start, etastart = etastart, :  
fitted probabilities numerically 0 or 1 occurred  
> summary(glm.01)
```

Call:



```
glm(formula = cbind(y1, y2) ~ pigment.1 + pigment.3 + wetwteyes,
     family = binomial(link = logit), data = data.mfa.df)
```

Deviance Residuals:

| Min        | 1Q         | Median    | 3Q        | Max       |
|------------|------------|-----------|-----------|-----------|
| -2.441e-04 | -2.107e-08 | 2.107e-08 | 2.107e-08 | 2.567e-04 |

Coefficients:

|             | Estimate | Std. Error | z value | Pr(> z ) |
|-------------|----------|------------|---------|----------|
| (Intercept) | 4100     | 533971     | 0.008   | 0.994    |
| pigment.1   | 1627     | 216712     | 0.008   | 0.994    |
| pigment.3   | -42130   | 5508443    | -0.008  | 0.994    |
| wetwteyes   | -108995  | 14252978   | -0.008  | 0.994    |

(Dispersion parameter for binomial family taken to be 1)

Null deviance: 4.6626e+01 on 36 degrees of freedom  
 Residual deviance: 1.4284e-07 on 33 degrees of freedom  
 AIC: 8

Number of Fisher Scoring iterations: 25

```
> glm.01 <- glm(cbind(y1,y2)~ sex+ pigment.1+wetwteyes + sex:pigment.1 +
sex:wetwteyes , family=binomial(link=logit), data=data.mfa.df)
```

Warning message:

```
In glm.fit(x = X, y = Y, weights = weights, start = start, etastart = etastart, :
fitted probabilities numerically 0 or 1 occurred
> summary(glm.01)
```

Call:

```
glm(formula = cbind(y1, y2) ~ sex + pigment.1 + wetwteyes + sex:pigment.1 +
sex:wetwteyes, family = binomial(link = logit), data = data.mfa.df)
```

Deviance Residuals:

| Min        | 1Q         | Median    | 3Q        | Max       |
|------------|------------|-----------|-----------|-----------|
| -1.380e+00 | -1.186e-02 | 3.787e-05 | 8.367e-02 | 1.638e+00 |

Coefficients:

|                | Estimate | Std. Error | z value | Pr(> z ) |
|----------------|----------|------------|---------|----------|
| (Intercept)    | 21.90    | 11.38      | 1.925   | 0.0543 . |
| sexm           | 45.47    | 17743.67   | 0.003   | 0.9980   |
| pigment.1      | -19.12   | 10.54      | -1.815  | 0.0696 . |
| wetwteyes      | -753.73  | 370.00     | -2.037  | 0.0416 * |
| sexm:pigment.1 | -21.58   | 38379.82   | -0.001  | 0.9996   |
| sexm:wetwteyes | -2725.61 | 3296547.26 | -0.001  | 0.9993   |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

(Dispersion parameter for binomial family taken to be 1)

Null deviance: 46.626 on 36 degrees of freedom  
Residual deviance: 10.459 on 31 degrees of freedom  
AIC: 22.459

Number of Fisher Scoring iterations: 19

```
>  
> glm.01 <- glm(cbind(y1,y2)~ wetwteyes, family=binomial(link=logit),  
data=data.mfa.df)  
> summary(glm.01)
```

Call:

```
glm(formula = cbind(y1, y2) ~ wetwteyes, family = binomial(link = logit),  
data = data.mfa.df)
```

Deviance Residuals:

| Min     | 1Q      | Median | 3Q     | Max    |
|---------|---------|--------|--------|--------|
| -2.5106 | -0.3836 | 0.3801 | 0.5488 | 2.3263 |

Coefficients:

|             | Estimate | Std. Error | z value | Pr(> z )     |
|-------------|----------|------------|---------|--------------|
| (Intercept) | 6.206    | 1.728      | 3.592   | 0.000328 *** |
| wetwteyes   | -645.454 | 198.052    | -3.259  | 0.001118 **  |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

(Dispersion parameter for binomial family taken to be 1)

Null deviance: 46.626 on 36 degrees of freedom  
Residual deviance: 26.735 on 35 degrees of freedom  
AIC: 30.735

Number of Fisher Scoring iterations: 5

```
> glm.01 <- glm(cbind(y1,y2)~ pigment.1+wetwteyes+CL,  
family=binomial(link=logit), data=data.mfa.df)
```

Warning messages:

1: In glm.fit(x = X, y = Y, weights = weights, start = start, etastart = etastart, :  
algorithm did not converge

2: In glm.fit(x = X, y = Y, weights = weights, start = start, etastart = etastart, :  
fitted probabilities numerically 0 or 1 occurred

```
> summary(glm.01)
```

Call:

```
glm(formula = cbind(y1, y2) ~ pigment.1 + wetwteyes + CL, family =  
binomial(link = logit),  
data = data.mfa.df)
```

Deviance Residuals:

| Min | 1Q | Median | 3Q | Max |
|-----|----|--------|----|-----|
|-----|----|--------|----|-----|

-2.883e-05 -2.107e-08 2.107e-08 2.107e-08 2.827e-05

Coefficients:

|             | Estimate   | Std. Error | z value   | Pr(> z ) |
|-------------|------------|------------|-----------|----------|
| (Intercept) | 4.518e+02  | 4.803e+05  | 0.001     | 1        |
| pigment.1   | -2.885e+02 | 3.227e+05  | -0.001    | 1        |
| wetwteyes   | -1.738e+03 | 1.049e+07  | -0.000166 | 1        |
| CL          | -1.904e+01 | 3.827e+04  | -0.000497 | 1        |

(Dispersion parameter for binomial family taken to be 1)

Null deviance: 4.6626e+01 on 36 degrees of freedom  
Residual deviance: 1.8272e-09 on 33 degrees of freedom  
AIC: 8

Number of Fisher Scoring iterations: 25

```
> glm.01 <- glm(cbind(y1,y2)~ pigment.1+wetwteyes+ED,  
family=binomial(link=logit), data=data.mfa.df)
```

Warning messages:

```
1: In glm.fit(x = X, y = Y, weights = weights, start = start, etastart = etastart, :  
algorithm did not converge  
2: In glm.fit(x = X, y = Y, weights = weights, start = start, etastart = etastart, :  
fitted probabilities numerically 0 or 1 occurred  
> summary(glm.01)
```

Call:

```
glm(formula = cbind(y1, y2) ~ pigment.1 + wetwteyes + ED, family =  
binomial(link = logit),  
data = data.mfa.df)
```

Deviance Residuals:

| Min        | 1Q         | Median    | 3Q        | Max       |
|------------|------------|-----------|-----------|-----------|
| -2.717e-05 | -2.107e-08 | 2.107e-08 | 2.107e-08 | 2.007e-05 |

Coefficients:

|             | Estimate | Std. Error | z value  | Pr(> z ) |
|-------------|----------|------------|----------|----------|
| (Intercept) | 561.3    | 551678.4   | 0.001    | 1        |
| pigment.1   | -226.4   | 272185.2   | -0.001   | 1        |
| wetwteyes   | 431.2    | 20231699.0 | 2.13e-05 | 1        |
| ED          | -213.3   | 233900.8   | -0.001   | 1        |

(Dispersion parameter for binomial family taken to be 1)

Null deviance: 4.6626e+01 on 36 degrees of freedom  
Residual deviance: 1.6179e-09 on 33 degrees of freedom  
AIC: 8

Number of Fisher Scoring iterations: 25

```
> glm.01 <- glm(cbind(y1,y2)~ pigment.1+ED, family=binomial(link=logit),
data=data.mfa.df)
```

Warning messages:

```
1: In glm.fit(x = X, y = Y, weights = weights, start = start, etastart = etastart, :
algorithm did not converge
```

```
2: In glm.fit(x = X, y = Y, weights = weights, start = start, etastart = etastart, :
fitted probabilities numerically 0 or 1 occurred
```

```
> summary(glm.01)
```

Call:

```
glm(formula = cbind(y1, y2) ~ pigment.1 + ED, family = binomial(link = logit),
data = data.mfa.df)
```

Deviance Residuals:

| Min        | 1Q         | Median    | 3Q        | Max       |
|------------|------------|-----------|-----------|-----------|
| -2.721e-05 | -2.107e-08 | 2.107e-08 | 2.107e-08 | 2.274e-05 |

Coefficients:

|             | Estimate | Std. Error | z value | Pr(> z ) |
|-------------|----------|------------|---------|----------|
| (Intercept) | 562.1    | 550132.4   | 0.001   | 1        |
| pigment.1   | -.223.8  | 237961.6   | -0.001  | 1        |
| ED          | -212.9   | 225266.5   | -0.001  | 1        |

(Dispersion parameter for binomial family taken to be 1)

Null deviance: 4.6626e+01 on 36 degrees of freedom

Residual deviance: 1.6356e-09 on 34 degrees of freedom

AIC: 6

Number of Fisher Scoring iterations: 25

```
>
```

```
> # final model 1 : pigment 1
```

```
>
```

```
> glm.01 <- glm(cbind(y1,y2)~ pigment.1+wetwteyes,
family=binomial(link=logit), data=data.mfa.df)
```

```
> summary(glm.01)
```

Call:

```
glm(formula = cbind(y1, y2) ~ pigment.1 + wetwteyes, family = binomial(link =
logit),
data = data.mfa.df)
```

Deviance Residuals:

| Min      | 1Q       | Median  | 3Q      | Max     |
|----------|----------|---------|---------|---------|
| -1.39339 | -0.03415 | 0.03133 | 0.07907 | 1.64272 |

Coefficients:

|             | Estimate | Std. Error | z value | Pr(> z ) |
|-------------|----------|------------|---------|----------|
| (Intercept) | 22.31    | 11.03      | 2.023   | 0.0431 * |

```
pigment.1    -19.50    10.22 -1.907 0.0565 .
wetwteyes    -765.84   362.27 -2.114 0.0345 *
```

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

(Dispersion parameter for binomial family taken to be 1)

Null deviance: 46.626 on 36 degrees of freedom  
Residual deviance: 10.481 on 34 degrees of freedom  
AIC: 16.481

Number of Fisher Scoring iterations: 8

```
>
> # final model 1 : pigment 1
>
> glm.01 <- glm(cbind(y1,y2)~ pigment.1+wetwteyes,
family=binomial(link=logit), data=data.mfa.df)
> summary(glm.01)
```

Call:

```
glm(formula = cbind(y1, y2) ~ pigment.1 + wetwteyes, family = binomial(link =
logit),
    data = data.mfa.df)
```

Deviance Residuals:

```
    Min      1Q   Median      3Q      Max
-1.39339 -0.03415  0.03133  0.07907  1.64272
```

Coefficients:

```
            Estimate Std. Error z value Pr(>|z|)
(Intercept)   22.31      11.03   2.023  0.0431 *
pigment.1     -19.50      10.22  -1.907  0.0565 .
wetwteyes     -765.84     362.27  -2.114  0.0345 *
```

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

(Dispersion parameter for binomial family taken to be 1)

Null deviance: 46.626 on 36 degrees of freedom  
Residual deviance: 10.481 on 34 degrees of freedom  
AIC: 16.481

Number of Fisher Scoring iterations: 8

```
> glm.pred.df <-
data.frame(cbind(glm.01$fitted.values, glm.01$residuals, glm.01$y, data.mfa.df$Age_
e_mth, data.mfa.df$sex, data.mfa.df$pigment.1,
+   data.mfa.df$wetwteyes, glm.01$linear.predictor))
>
```

```

> names(glm.pred.df) <-
c("fv","resids","y","Age_mth","sex","pigment.1","wetwteyes","lp")
>
> # sort glm.pred.df
>
> glm.pred.s.df <- glm.pred.df[order(glm.pred.df$lp),]
>
> # find optimal cutpoint on lp for %correct age 12 month + (100-(%incorrect age
12 + %incorrect age 36 mnth))
>
> N12 <- sum(glm.pred.s.df$y)
> Ndim <- dim(glm.pred.s.df)[1]
> N36 <- Ndim-N12
> c(N12,N36,Ndim)
[1] 25 12 37
> Cor12 <- rep(0,Ndim)
> Cor36 <- rep(0,Ndim)
>
>
> for (j in 1:Ndim) {
+ Cor12[j] <- sum(as.integer(glm.pred.s.df$y==1 & (seq(1,Ndim)>=j)))
+ Cor36[j] <- sum(as.integer(glm.pred.s.df$y==0 & (seq(1,Ndim)<j)))}
>
> Cor12P <- 100*Cor12/N12
> Cor36P <- 100*Cor36/N36
> CorB <- Cor12P+Cor36P
> glm.pred.s.df$Cor12P <- Cor12P
> glm.pred.s.df$Cor36P <- Cor36P
> glm.pred.s.df$CorB <- CorB
>
> #edit(glm.pred.s.df)
>
> glm.pred.opt.df <- glm.pred.s.df[order(glm.pred.s.df$CorB),]
>
> glm.pred.opt.df[Ndim,]
      fv  resids y Age_mth sex pigment.1 wetwteyes   lp Cor12P Cor36P
8 0.7962806 1.255839 1   12   1 0.8975459   0.0045 1.363208   92   100
      CorB
8 192
> # include just pigment 1 in LP
>
> glm.01 <- glm(cbind(y1,y2)~ pigment.1, family=binomial(link=logit),
data=data.mfa.df)
> summary(glm.01)

```

Call:

```

glm(formula = cbind(y1, y2) ~ pigment.1, family = binomial(link = logit),
     data = data.mfa.df)

```

Deviance Residuals:

| Min      | 1Q       | Median  | 3Q      | Max     |
|----------|----------|---------|---------|---------|
| -2.19432 | -0.08578 | 0.28057 | 0.58332 | 1.86279 |

Coefficients:

|             | Estimate | Std. Error | z value | Pr(> z )   |
|-------------|----------|------------|---------|------------|
| (Intercept) | 9.798    | 3.116      | 3.144   | 0.00167 ** |
| pigment.1   | -12.634  | 4.248      | -2.974  | 0.00294 ** |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

(Dispersion parameter for binomial family taken to be 1)

Null deviance: 46.626 on 36 degrees of freedom  
 Residual deviance: 24.162 on 35 degrees of freedom  
 AIC: 28.162

Number of Fisher Scoring iterations: 6

```
>
> glm.pred.df <-
data.frame(cbind(glm.01$fitted.values,glm.01$residuals,glm.01$y,data.mfa.df$Age_
e_mth,data.mfa.df$sex,data.mfa.df$pigment.1,
+ data.mfa.df$wetwteyes,glm.01$linear.predictor))
>
> names(glm.pred.df) <-
c("fv","resids","y","Age_mth","sex","pigment.1","wetwteyes","lp")
>
> # sort glm.pred.df
>
> glm.pred.s.df <- glm.pred.df[order(glm.pred.df$lp),]
>
> # find optimal cutpoint on lp for %correct age 12 month + (100-(%incorrect age
12 + %incorrect age 36 mnth))
>
> N12 <- sum(glm.pred.s.df$y)
> Ndim <- dim(glm.pred.s.df)[1]
> N36 <- Ndim-N12
> c(N12,N36,Ndim)
[1] 25 12 37
> Cor12 <- rep(0,Ndim)
> Cor36 <- rep(0,Ndim)
>
>
> for (j in 1:Ndim) {
+ Cor12[j] <- sum(as.integer(glm.pred.s.df$y==1 & (seq(1,Ndim)>=j)))
+ Cor36[j] <- sum(as.integer(glm.pred.s.df$y==0 & (seq(1,Ndim)<j))))}
>
> Cor12P <- 100*Cor12/N12
> Cor36P <- 100*Cor36/N36
> CorB <- Cor12P+Cor36P
```

```

> glm.pred.s.df$Cor12P <- Cor12P
> glm.pred.s.df$Cor36P <- Cor36P
> glm.pred.s.df$CorB <- CorB
>
> #edit(glm.pred.s.df)
>
> glm.pred.opt.df <- glm.pred.s.df[order(glm.pred.s.df$CorB),]
>
> glm.pred.opt.df[Ndim,]
      fv  resids y Age_mth sex pigment.1 wetwteyes      lp Cor12P
14 0.7107453 1.406974 1     12 1 0.7044182  0.0065 0.8990067   92
      Cor36P  CorB
14 83.33333 175.3333
>
> # final model 2 : pigment.3
>
> # include just pigment.3 in LP
>
> glm.01 <- glm(cbind(y1,y2)~ sex+ pigment.3+wetwteyes + sex:pigment.3 +
sex:wetwteyes , family=binomial(link=logit), data=data.mfa.df)
Warning message:
In glm.fit(x = X, y = Y, weights = weights, start = start, etastart = etastart, :
fitted probabilities numerically 0 or 1 occurred
> summary(glm.01)

```

Call:

```

glm(formula = cbind(y1, y2) ~ sex + pigment.3 + wetwteyes + sex:pigment.3 +
sex:wetwteyes, family = binomial(link = logit), data = data.mfa.df)

```

Deviance Residuals:

| Min        | 1Q         | Median    | 3Q        | Max       |
|------------|------------|-----------|-----------|-----------|
| -1.228e+00 | -8.141e-06 | 3.743e-06 | 4.415e-04 | 1.321e+00 |

Coefficients:

|                | Estimate | Std. Error | z value   | Pr(> z ) |
|----------------|----------|------------|-----------|----------|
| (Intercept)    | 54.34    | 61.24      | 0.887     | 0.375    |
| sexm           | 20.06    | 32565.36   | 0.001     | 1.000    |
| pigment.3      | -365.85  | 429.10     | -0.853    | 0.394    |
| wetwteyes      | -1751.18 | 1850.29    | -0.946    | 0.344    |
| sexm:pigment.3 | -31.99   | 437842.28  | -0.000073 | 1.000    |
| sexm:wetwteyes | -1494.51 | 6687094.37 | -0.000223 | 1.000    |

(Dispersion parameter for binomial family taken to be 1)

Null deviance: 46.626 on 36 degrees of freedom  
Residual deviance: 3.402 on 31 degrees of freedom  
AIC: 15.402

Number of Fisher Scoring iterations: 20



```
> glm.01 <- glm(cbind(y1,y2)~ pigment.3+wetwteyes,
family=binomial(link=logit), data=data.mfa.df)
Warning message:
In glm.fit(x = X, y = Y, weights = weights, start = start, etastart = etastart, :
  fitted probabilities numerically 0 or 1 occurred
> summary(glm.01)
```

Call:

```
glm(formula = cbind(y1, y2) ~ pigment.3 + wetwteyes, family = binomial(link =
logit),
  data = data.mfa.df)
```

Deviance Residuals:

| Min        | 1Q         | Median    | 3Q        | Max       |
|------------|------------|-----------|-----------|-----------|
| -1.228e+00 | -8.136e-06 | 1.047e-05 | 7.116e-04 | 1.321e+00 |

Coefficients:

|             | Estimate | Std. Error | z value | Pr(> z ) |
|-------------|----------|------------|---------|----------|
| (Intercept) | 54.35    | 61.23      | 0.888   | 0.375    |
| pigment.3   | -365.87  | 429.02     | -0.853  | 0.394    |
| wetwteyes   | -1751.25 | 1849.92    | -0.947  | 0.344    |

(Dispersion parameter for binomial family taken to be 1)

Null deviance: 46.626 on 36 degrees of freedom  
Residual deviance: 3.402 on 34 degrees of freedom  
AIC: 9.402

Number of Fisher Scoring iterations: 12

```
>
> #plot(glm.01)
>
> glm.pred.df <-
data.frame(cbind(glm.01$fitted.values,glm.01$residuals,glm.01$y,data.mfa.df$Age_
mth,data.mfa.df$sex,data.mfa.df$pigment.3,
+ data.mfa.df$wetwteyes,glm.01$linear.predictor))
>
> names(glm.pred.df) <-
c("fv","resids","y","Age_mth","sex","pigment.3","wetwteyes","lp")
>
> # sort glm.pred.df
>
> glm.pred.s.df <- glm.pred.df[order(glm.pred.df$lp),]
>
> # find optimal cutpoint on lp for %correct age 12 month + (100-(%incorrect age
12 + %incorrect age 36 mnth))
>
> N12 <- sum(glm.pred.s.df$y)
> Ndim <- dim(glm.pred.s.df)[1]
```

```

> N36 <- Ndim-N12
> c(N12,N36,Ndim)
[1] 25 12 37
> Cor12 <- rep(0,Ndim)
> Cor36 <- rep(0,Ndim)
>
>
> for (j in 1:Ndim) {
+ Cor12[j] <- sum(as.integer(glm.pred.s.df$y==1 & (seq(1,Ndim)>=j)))
+ Cor36[j] <- sum(as.integer(glm.pred.s.df$y==0 & (seq(1,Ndim)<j)))}
>
> Cor12P <- 100*Cor12/N12
> Cor36P <- 100*Cor36/N36
> CorB <- Cor12P+Cor36P
> glm.pred.s.df$Cor12P <- Cor12P
> glm.pred.s.df$Cor36P <- Cor36P
> glm.pred.s.df$CorB <- CorB
>
> #edit(glm.pred.s.df)
>
> glm.pred.opt.df <- glm.pred.s.df[order(glm.pred.s.df$CorB),]
>
> glm.pred.opt.df[Ndim,]
      fv  resid y Age_mth sex pigment.3 wetwteyes      lp Cor12P Cor36P
15 0.990126 1.009972 1    12 1 0.07036922 0.0137 4.607937  96  100
      CorB
15 196
>
> # include just pigment.3 in LP
> glm.01 <- glm(cbind(y1,y2)~ pigment.3, family=binomial(link=logit),
data=data.mfa.df)
> summary(glm.01)

```

Call:

```
glm(formula = cbind(y1, y2) ~ pigment.3, family = binomial(link = logit),
    data = data.mfa.df)
```

Deviance Residuals:

| Min      | 1Q       | Median  | 3Q      | Max     |
|----------|----------|---------|---------|---------|
| -2.22827 | -0.01726 | 0.05390 | 0.24655 | 1.42610 |

Coefficients:

|             | Estimate | Std. Error | z value | Pr(> z )   |
|-------------|----------|------------|---------|------------|
| (Intercept) | 13.695   | 5.069      | 2.702   | 0.00690 ** |
| pigment.3   | -134.304 | 50.589     | -2.655  | 0.00794 ** |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

(Dispersion parameter for binomial family taken to be 1)

Null deviance: 46.626 on 36 degrees of freedom  
 Residual deviance: 12.490 on 35 degrees of freedom  
 AIC: 16.49

Number of Fisher Scoring iterations: 7

```
> #plot(glm.01)
>
> glm.pred.df <-
data.frame(cbind(glm.01$fitted.values,glm.01$residuals,glm.01$y,data.mfa.df$Age_
mth,data.mfa.df$sex,data.mfa.df$pigment.3,
+ data.mfa.df$wetwteyes,glm.01$linear.predictor))
>
> names(glm.pred.df) <-
c("fv","resids","y","Age_mth","sex","pigment.3","wetwteyes","lp")
>
> # sort glm.pred.df
>
> glm.pred.s.df <- glm.pred.df[order(glm.pred.df$lp),]
>
> # find optimal cutpoint on lp for %correct age 12 month + (100-(%incorrect age
12 + %incorrect age 36 mnth))
>
> N12 <- sum(glm.pred.s.df$y)
> Ndim <- dim(glm.pred.s.df)[1]
> N36 <- Ndim-N12
> c(N12,N36,Ndim)
[1] 25 12 37
> Cor12 <- rep(0,Ndim)
> Cor36 <- rep(0,Ndim)
>
>
> for (j in 1:Ndim) {
+ Cor12[j] <- sum(as.integer(glm.pred.s.df$y==1 & (seq(1,Ndim)>=j)))
+ Cor36[j] <- sum(as.integer(glm.pred.s.df$y==0 & (seq(1,Ndim)<j))))
>
> Cor12P <- 100*Cor12/N12
> Cor36P <- 100*Cor36/N36
> CorB <- Cor12P+Cor36P
> glm.pred.s.df$Cor12P <- Cor12P
> glm.pred.s.df$Cor36P <- Cor36P
> glm.pred.s.df$CorB <- CorB
>
> #edit(glm.pred.s.df)
>
> glm.pred.opt.df <- glm.pred.s.df[order(glm.pred.s.df$CorB),]
>
> glm.pred.opt.df[Ndim,]
      fv  resids y Age_mth sex pigment.3 wetwteyes   lp Cor12P
8 0.3617224 2.764551 1    12  1 0.1062024  0.0045 -0.5678963  100
```

```

Cor36P CorB
8 91.66667 191.6667
>
> # now use carapace length instead of pigments
>
> glm.01 <- glm(cbind(y1,y2)~ CL+wetwteyes, family=binomial(link=logit),
data=data.mfa.df)
> summary(glm.01)

```

Call:

```

glm(formula = cbind(y1, y2) ~ CL + wetwteyes, family = binomial(link = logit),
    data = data.mfa.df)

```

Deviance Residuals:

| Min     | 1Q      | Median | 3Q     | Max    |
|---------|---------|--------|--------|--------|
| -1.9402 | -0.1201 | 0.2577 | 0.4391 | 1.7658 |

Coefficients:

|             | Estimate | Std. Error | z value | Pr(> z ) |
|-------------|----------|------------|---------|----------|
| (Intercept) | 17.753   | 7.014      | 2.531   | 0.0114 * |
| CL          | -1.269   | 0.659      | -1.925  | 0.0542 . |
| wetwteyes   | -338.361 | 256.724    | -1.318  | 0.1875   |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

(Dispersion parameter for binomial family taken to be 1)

Null deviance: 46.626 on 36 degrees of freedom  
Residual deviance: 20.587 on 34 degrees of freedom  
AIC: 26.587

Number of Fisher Scoring iterations: 6

```

>
> # use just CL
>
> glm.01 <- glm(cbind(y1,y2)~ CL, family=binomial(link=logit),
data=data.mfa.df)
> summary(glm.01)

```

Call:

```

glm(formula = cbind(y1, y2) ~ CL, family = binomial(link = logit),
    data = data.mfa.df)

```

Deviance Residuals:

| Min     | 1Q      | Median | 3Q     | Max    |
|---------|---------|--------|--------|--------|
| -2.6358 | -0.1693 | 0.2019 | 0.5061 | 1.1256 |

Coefficients:

|  | Estimate | Std. Error | z value | Pr(> z ) |
|--|----------|------------|---------|----------|
|--|----------|------------|---------|----------|

```
(Intercept) 19.9492  7.0157  2.843  0.00446 **
CL          -1.6945  0.6179 -2.742  0.00610 **
```

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

(Dispersion parameter for binomial family taken to be 1)

Null deviance: 46.626 on 36 degrees of freedom  
Residual deviance: 22.544 on 35 degrees of freedom  
AIC: 26.544

Number of Fisher Scoring iterations: 6

```
>
>
> #plot(glm.01)
>
> glm.pred.df <-
data.frame(cbind(glm.01$fitted.values, glm.01$residuals, glm.01$y, data.mfa.df$Age_
e_mth, data.mfa.df$sex, data.mfa.df$CL,
+   data.mfa.df$wetwteyes, glm.01$linear.predictor))
>
> names(glm.pred.df) <-
c("fv", "resids", "y", "Age_mth", "sex", "CL", "wetwteyes", "lp")
>
> # sort glm.pred.df
>
> glm.pred.s.df <- glm.pred.df[order(glm.pred.df$lp),]
>
> # find optimal cutpoint on lp for %correct age 12 month + (100-(%incorrect age
12 + %incorrect age 36 mnth))
>
> N12 <- sum(glm.pred.s.df$y)
> Ndim <- dim(glm.pred.s.df)[1]
> N36 <- Ndim-N12
> c(N12, N36, Ndim)
[1] 25 12 37
> Cor12 <- rep(0, Ndim)
> Cor36 <- rep(0, Ndim)
>
>
> for (j in 1:Ndim) {
+ Cor12[j] <- sum(as.integer(glm.pred.s.df$y==1 & (seq(1, Ndim)>=j)))
+ Cor36[j] <- sum(as.integer(glm.pred.s.df$y==0 & (seq(1, Ndim)<j))))
>
> Cor12P <- 100*Cor12/N12
> Cor36P <- 100*Cor36/N36
> CorB <- Cor12P+Cor36P
> glm.pred.s.df$Cor12P <- Cor12P
> glm.pred.s.df$Cor36P <- Cor36P
```

```

> glm.pred.s.df$CorB <- CorB
>
> #edit(glm.pred.s.df)
>
> glm.pred.opt.df <- glm.pred.s.df[order(glm.pred.s.df$CorB),]
>
> glm.pred.opt.df[Ndim,]
      fv  resid y Age_mth sex  CL wetwteyes  lp Cor12P Cor36P
4 0.5307287 1.884202 1  12  1 11.70021  0.0066 0.1230700  100  75
  CorB
4 175
>
>
> glm.01 <- glm(cbind(y1,y2)~ sex+CL+sex:CL, family=binomial(link=logit),
data=data.mfa.df)
> summary(glm.01)

```

Call:

```

glm(formula = cbind(y1, y2) ~ sex + CL + sex:CL, family = binomial(link = logit),
    data = data.mfa.df)

```

Deviance Residuals:

| Min      | 1Q       | Median  | 3Q      | Max     |
|----------|----------|---------|---------|---------|
| -2.04545 | -0.03607 | 0.26173 | 0.45090 | 1.13887 |

Coefficients:

|             | Estimate | Std. Error | z value | Pr(> z ) |
|-------------|----------|------------|---------|----------|
| (Intercept) | 33.860   | 17.599     | 1.924   | 0.0544 . |
| sexm        | -13.862  | 24.865     | -0.557  | 0.5772   |
| CL          | -2.886   | 1.542      | -1.872  | 0.0612 . |
| sexm:CL     | 1.029    | 2.358      | 0.437   | 0.6624   |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

(Dispersion parameter for binomial family taken to be 1)

Null deviance: 46.626 on 36 degrees of freedom  
Residual deviance: 19.719 on 33 degrees of freedom  
AIC: 27.719

Number of Fisher Scoring iterations: 7

```

>
> glm.01 <- glm(cbind(y1,y2)~ sex+pigment.1+sex:pigment.1+CL+sex:CL,
family=binomial(link=logit), data=data.mfa.df)
Warning messages:
1: In glm.fit(x = X, y = Y, weights = weights, start = start, etastart = etastart, :
  algorithm did not converge
2: In glm.fit(x = X, y = Y, weights = weights, start = start, etastart = etastart, :
  fitted probabilities numerically 0 or 1 occurred

```

```
> summary(glm.01)
```

Call:

```
glm(formula = cbind(y1, y2) ~ sex + pigment.1 + sex:pigment.1 +  
CL + sex:CL, family = binomial(link = logit), data = data.mfa.df)
```

Deviance Residuals:

| Min        | 1Q         | Median    | 3Q        | Max       |
|------------|------------|-----------|-----------|-----------|
| -2.302e-05 | -2.107e-08 | 2.107e-08 | 2.107e-08 | 2.487e-05 |

Coefficients:

|                | Estimate | Std. Error | z value   | Pr(> z ) |
|----------------|----------|------------|-----------|----------|
| (Intercept)    | 496.42   | 622094.29  | 0.001     | 1        |
| sexm           | -357.50  | 1312827.29 | -2.72e-04 | 1        |
| pigment.1      | -318.05  | 433160.38  | -0.001    | 1        |
| CL             | -22.11   | 46240.03   | -4.78e-04 | 1        |
| sexm:pigment.1 | 235.78   | 999023.96  | 2.36e-04  | 1        |
| sexm:CL        | 15.28    | 153494.96  | 9.95e-05  | 1        |

(Dispersion parameter for binomial family taken to be 1)

Null deviance: 4.6626e+01 on 36 degrees of freedom  
Residual deviance: 1.2706e-09 on 31 degrees of freedom  
AIC: 12

Number of Fisher Scoring iterations: 25

```
>
```

## Appendix IV: Multivariate analysis of factors affecting pigment accumulation

---

### *R code and output*

R version 2.7.0 (2008-04-22)

Copyright (C) 2008 The R Foundation for Statistical Computing

ISBN 3-900051-07-0

R is free software and comes with ABSOLUTELY NO WARRANTY.

You are welcome to redistribute it under certain conditions.

Type 'license()' or 'licence()' for distribution details.

Natural language support but running in an English locale

R is a collaborative project with many contributors.

Type 'contributors()' for more information and

'citation()' on how to cite R or R packages in publications.

Type 'demo()' for some demos, 'help()' for on-line help, or

'help.start()' for an HTML browser interface to help.

Type 'q()' to quit R.

```
> # Angela's pigment analyses
> # part 1 Cumulative pigment vs age
>
> library(lattice)
>
> data.df <- read.csv(file="Part3.csv")
>
> treat.df <- read.csv(file="treat.csv")
>
> data.df <- cbind(data.df,treat.df)
>
> summary(data.df)
```

|                                         | Group | CL             | TL           |
|-----------------------------------------|-------|----------------|--------------|
| Cold, mixed diet, no stress, 7 months : | 11    | Min. : 6.765   | Mode:logical |
| Cold, limited diet, no stress, 1 month: | 10    | 1st Qu.: 8.857 | NA's:262     |
| Cold, limited diet, stress, 1 month :   | 10    | Median : 9.449 |              |
| Cold, mixed diet, no stress, 1 month :  | 10    | Mean : 9.440   |              |
| Cold, mixed diet, no stress, 4 months : | 10    | 3rd Qu.:10.044 |              |
| Cold, mixed diet, stress, 1 month :     | 10    | Max. :12.416   |              |
| (Other)                                 | :201  |                |              |

|              | ED | wetwteyes       | sex   | FAP_Ex280Em625 | FAP_Ex355Em510 |
|--------------|----|-----------------|-------|----------------|----------------|
| 1.491512676: | 6  | Min. :0.00240   | f: 99 | Min. :0.5000   | Min. :0.1000   |
| 1.356617176: | 3  | 1st Qu.:0.00390 | j: 51 | 1st Qu.:0.8000 | 1st Qu.:0.2000 |
| 1.414676494: | 3  | Median :0.00485 | m:112 | Median :0.9000 | Median :0.2000 |
| 1.514958657: | 3  | Mean :0.00682   |       | Mean :0.9706   | Mean :0.2244   |



1.5916251 : 3 3rd Qu.:0.00680 3rd Qu.:1.1000 3rd Qu.:0.3000  
 1.302206013: 2 Max. :0.14300 Max. :1.9000 Max. :1.5000  
 (Other) :242  
 FAP\_Ex463Em620 protein pigment.1 pigment.2  
 Min. :0.0000 Min. :0.400 Min. :0.3290 Min. :0.05041  
 1st Qu.:0.1000 1st Qu.:1.000 1st Qu.:0.6753 1st Qu.:0.13802  
 Median :0.1000 Median :1.200 Median :0.8010 Median :0.17486  
 Mean :0.1321 Mean :1.218 Mean :0.8473 Mean :0.20066  
 3rd Qu.:0.2000 3rd Qu.:1.400 3rd Qu.:0.9530 3rd Qu.:0.23060  
 Max. :0.4000 Max. :3.000 Max. :1.9118 Max. :1.66625

pigment.3 Temp.f Diet.f Stress.f  
 Min. :0.03016 Cold:142 limited diet: 64 no stress:128  
 1st Qu.:0.08955 Warm:120 mixed diet :100 stress :134  
 Median :0.10862 phyto diet : 98  
 Mean :0.12098  
 3rd Qu.:0.14009  
 Max. :0.37760

Age.f  
 1 month :120  
 4 months: 90  
 7 months: 52

```
>
> # analyse pigment.1
>
> # fit linear model with main effects only
>
> lm.01 <- lm(formula=pigment.1 ~ Age.f+sex+Temp.f+
+ Diet.f+Stress.f, data=data.df)
>
> anova(lm.01)
Analysis of Variance Table
```

```
Response: pigment.1
      Df Sum Sq Mean Sq F value Pr(>F)
Age.f   2  0.9684  0.4842  7.4608 0.0007108 ***
sex     2  0.1358  0.0679  1.0463 0.3527373
Temp.f   1  0.0221  0.0221  0.3407 0.5599351
Diet.f   2  0.2047  0.1023  1.5768 0.2086636
Stress.f 1  0.0917  0.0917  1.4125 0.2357612
Residuals 253 16.4200  0.0649
```

```
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
> summary(lm.01)
```

Call:

```
lm(formula = pigment.l ~ Age.f + sex + Temp.f + Diet.f + Stress.f,  
    data = data.df)
```

Residuals:

| Min      | 1Q       | Median   | 3Q      | Max     |
|----------|----------|----------|---------|---------|
| -0.61543 | -0.15989 | -0.05302 | 0.12060 | 0.99309 |

Coefficients:

|                   | Estimate | Std. Error | t value | Pr(> t )    |
|-------------------|----------|------------|---------|-------------|
| (Intercept)       | 0.94639  | 0.05123    | 18.474  | < 2e-16 *** |
| Age.f 4 months    | -0.11982 | 0.03834    | -3.125  | 0.00198 **  |
| Age.f 7 months    | 0.05029  | 0.04681    | 1.074   | 0.28366     |
| sexj              | -0.07397 | 0.04947    | -1.495  | 0.13611     |
| sexm              | -0.04371 | 0.03599    | -1.215  | 0.22569     |
| Temp.fWarm        | -0.01894 | 0.03204    | -0.591  | 0.55507     |
| Diet.f mixed diet | -0.04019 | 0.04259    | -0.944  | 0.34625     |
| Diet.f phyto diet | 0.02273  | 0.04176    | 0.544   | 0.58668     |
| Stress.f stress   | -0.03774 | 0.03175    | -1.188  | 0.23576     |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 0.2548 on 253 degrees of freedom

Multiple R-squared: 0.07974, Adjusted R-squared: 0.05064

F-statistic: 2.74 on 8 and 253 DF, p-value: 0.006465

>

> # fit linear model with main effects + first-order interactions

>

> lm.02 <- lm(formula=pigment.l ~ Age.f+sex+Temp.f+

+ Diet.f+Stress.f+Age.f:sex+Age.f:Temp.f+Age.f:Diet.f+Age.f:Stress.f+

+ sex:Temp.f+sex:Diet.f+sex:Stress.f+Temp.f:Diet.f+Temp.f:Stress.f+

+ Diet.f:Stress.f, data=data.df)

>

> anova(lm.02)

Analysis of Variance Table

Response: pigment.l

|                | Df | Sum Sq | Mean Sq | F value | Pr(>F)        |
|----------------|----|--------|---------|---------|---------------|
| Age.f          | 2  | 0.9684 | 0.4842  | 9.5263  | 0.0001061 *** |
| sex            | 2  | 0.1358 | 0.0679  | 1.3360  | 0.2649340     |
| Temp.f         | 1  | 0.0221 | 0.0221  | 0.4350  | 0.5101869     |
| Diet.f         | 2  | 0.2047 | 0.1023  | 2.0133  | 0.1359040     |
| Stress.f       | 1  | 0.0917 | 0.0917  | 1.8035  | 0.1806187     |
| Age.f:sex      | 3  | 0.0958 | 0.0319  | 0.6283  | 0.5974392     |
| Age.f:Temp.f   | 2  | 0.9227 | 0.4613  | 9.0762  | 0.0001610 *** |
| Age.f:Diet.f   | 4  | 0.4785 | 0.1196  | 2.3534  | 0.0548028 .   |
| Age.f:Stress.f | 2  | 0.0091 | 0.0045  | 0.0894  | 0.9145132     |
| sex:Temp.f     | 2  | 0.0959 | 0.0479  | 0.9430  | 0.3909706     |
| sex:Diet.f     | 4  | 0.3299 | 0.0825  | 1.6227  | 0.1693550     |

```
sex:Stress.f    2 0.1978 0.0989 1.9453 0.1452942
Temp.f:Diet.f  2 2.3991 1.1995 23.5995 4.8e-10 ***
Temp.f:Stress.f 1 0.0177 0.0177 0.3489 0.5553005
Diet.f:Stress.f 2 0.2337 0.1168 2.2987 0.1027049
Residuals      229 11.6399 0.0508
```

---

```
Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
> summary(lm.02)
```

Call:

```
lm(formula = pigment.l ~ Age.f + sex + Temp.f + Diet.f + Stress.f +
    Age.f:sex + Age.f:Temp.f + Age.f:Diet.f + Age.f:Stress.f +
    sex:Temp.f + sex:Diet.f + sex:Stress.f + Temp.f:Diet.f +
    Temp.f:Stress.f + Diet.f:Stress.f, data = data.df)
```

Residuals:

```
Min      1Q  Median      3Q      Max
-0.79911 -0.12893 -0.01677  0.09665  0.84845
```

Coefficients: (1 not defined because of singularities)

|                                  | Estimate  | Std. Error | t value | Pr(> t )     |
|----------------------------------|-----------|------------|---------|--------------|
| (Intercept)                      | 0.720651  | 0.089109   | 8.087   | 3.54e-14 *** |
| Age.f 4 months                   | -0.005229 | 0.096041   | -0.054  | 0.956626     |
| Age.f 7 months                   | 0.009244  | 0.142309   | 0.065   | 0.948266     |
| sexj                             | -0.014123 | 0.109145   | -0.129  | 0.897155     |
| sexm                             | 0.027955  | 0.089520   | 0.312   | 0.755110     |
| Temp.fWarm                       | 0.374616  | 0.089463   | 4.187   | 4.03e-05 *** |
| Diet.f mixed diet                | 0.093751  | 0.101342   | 0.925   | 0.355890     |
| Diet.f phyto diet                | 0.243235  | 0.099568   | 2.443   | 0.015326 *   |
| Stress.f stress                  | 0.051136  | 0.091819   | 0.557   | 0.578128     |
| Age.f 4 months:sexj              | 0.135059  | 0.110818   | 1.219   | 0.224196     |
| Age.f 7 months:sexj              | NA        | NA         | NA      | NA           |
| Age.f 4 months:sexm              | 0.002972  | 0.077122   | 0.039   | 0.969294     |
| Age.f 7 months:sexm              | 0.034998  | 0.086770   | 0.403   | 0.687075     |
| Age.f 4 months:Temp.fWarm        | -0.014985 | 0.070059   | -0.214  | 0.830824     |
| Age.f 7 months:Temp.fWarm        | -0.363769 | 0.099719   | -3.648  | 0.000327 *** |
| Age.f 4 months:Diet.f mixed diet | -0.165612 | 0.091324   | -1.813  | 0.071070 .   |
| Age.f 7 months:Diet.f mixed diet | 0.080502  | 0.141046   | 0.571   | 0.568730     |
| Age.f 4 months:Diet.f phyto diet | -0.067191 | 0.087608   | -0.767  | 0.443898     |
| Age.f 7 months:Diet.f phyto diet | 0.229970  | 0.145099   | 1.585   | 0.114364     |
| Age.f 4 months:Stress.f stress   | -0.081217 | 0.068892   | -1.179  | 0.239661     |
| Age.f 7 months:Stress.f stress   | 0.005749  | 0.098471   | 0.058   | 0.953493     |
| sexj:Temp.fWarm                  | -0.179828 | 0.093275   | -1.928  | 0.055100 .   |
| sexm:Temp.fWarm                  | -0.059762 | 0.066406   | -0.900  | 0.369090     |
| sexj:Diet.f mixed diet           | -0.021188 | 0.115892   | -0.183  | 0.855098     |
| sexm:Diet.f mixed diet           | -0.038753 | 0.090488   | -0.428  | 0.668857     |
| sexj:Diet.f phyto diet           | 0.211119  | 0.111519   | 1.893   | 0.059603 .   |
| sexm:Diet.f phyto diet           | -0.034518 | 0.089625   | -0.385  | 0.700495     |
| sexj:Stress.f stress             | -0.166687 | 0.089558   | -1.861  | 0.063995 .   |
| sexm:Stress.f stress             | -0.047390 | 0.066542   | -0.712  | 0.477075     |

```
Temp.fWarm:Diet.f mixed diet -0.300837 0.079629 -3.778 0.000202 ***
Temp.fWarm:Diet.f phyto diet -0.501156 0.077111 -6.499 4.99e-10 ***
Temp.fWarm:Stress.f stress 0.050033 0.062054 0.806 0.420913
Diet.f mixed diet:Stress.f stress 0.102362 0.077664 1.318 0.188818
Diet.f phyto diet:Stress.f stress -0.042931 0.077822 -0.552 0.581723
```

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 0.2255 on 229 degrees of freedom  
Multiple R-squared: 0.3476, Adjusted R-squared: 0.2565  
F-statistic: 3.813 on 32 and 229 DF, p-value: 1.569e-09

>

> # reduced model

>

```
> lm.03 <- lm(formula=pigment.1 ~ Age.f+Temp.f+
+ Diet.f+Age.f:Temp.f+Age.f:Diet.f+Temp.f:Diet.f, data=data.df)
```

>

> anova(lm.03)

Analysis of Variance Table

Response: pigment.1

|               | Df  | Sum Sq  | Mean Sq | F value | Pr(>F)        |
|---------------|-----|---------|---------|---------|---------------|
| Age.f         | 2   | 0.9684  | 0.4842  | 9.1248  | 0.0001500 *** |
| Temp.f        | 1   | 0.0189  | 0.0189  | 0.3555  | 0.5515358     |
| Diet.f        | 2   | 0.1694  | 0.0847  | 1.5966  | 0.2046662     |
| Age.f:Temp.f  | 2   | 0.9922  | 0.4961  | 9.3491  | 0.0001218 *** |
| Age.f:Diet.f  | 4   | 0.4941  | 0.1235  | 2.3275  | 0.0568408 .   |
| Temp.f:Diet.f | 2   | 2.0393  | 1.0196  | 19.2147 | 1.745e-08 *** |
| Residuals     | 248 | 13.1604 | 0.0531  |         |               |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

> summary(lm.03)

Call:

```
lm(formula = pigment.1 ~ Age.f + Temp.f + Diet.f + Age.f:Temp.f +
Age.f:Diet.f + Temp.f:Diet.f, data = data.df)
```

Residuals:

| Min      | 1Q       | Median   | 3Q      | Max     |
|----------|----------|----------|---------|---------|
| -0.79843 | -0.14433 | -0.02091 | 0.10197 | 0.92846 |

Coefficients:

|                   | Estimate | Std. Error | t value | Pr(> t )     |
|-------------------|----------|------------|---------|--------------|
| (Intercept)       | 0.70255  | 0.04780    | 14.697  | < 2e-16 ***  |
| Age.f 4 months    | 0.02194  | 0.07108    | 0.309   | 0.75781      |
| Age.f 7 months    | 0.06481  | 0.12734    | 0.509   | 0.61122      |
| Temp.fWarm        | 0.25939  | 0.06192    | 4.189   | 3.89e-05 *** |
| Diet.f mixed diet | 0.13466  | 0.06424    | 2.096   | 0.03708 *    |
| Diet.f phyto diet | 0.29794  | 0.06379    | 4.671   | 4.92e-06 *** |

```

Age.f 4 months:Temp.fWarm      0.04227  0.06551  0.645  0.51941
Age.f 7 months:Temp.fWarm      -0.25722  0.08054 -3.194  0.00159 **
Age.f 4 months:Diet.f mixed diet -0.19654  0.08481 -2.317  0.02130 *
Age.f 7 months:Diet.f mixed diet  0.08136  0.13354  0.609  0.54292
Age.f 4 months:Diet.f phyto diet -0.17074  0.08144 -2.096  0.03706 *
Age.f 7 months:Diet.f phyto diet  0.13712  0.13743  0.998  0.31938
Temp.fWarm:Diet.f mixed diet    -0.20165  0.07678 -2.626  0.00917 **
Temp.fWarm:Diet.f phyto diet    -0.45565  0.07525 -6.055  5.16e-09 ***

```

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 0.2304 on 248 degrees of freedom  
Multiple R-squared: 0.2624, Adjusted R-squared: 0.2238  
F-statistic: 6.787 on 13 and 248 DF, p-value: 3.843e-11

>

> # analyse pigment.2

>

> # fit linear model with main effects only

>

> lm.01 <- lm(formula=pigment.2 ~ Age.f+sex+Temp.f+  
+ Diet.f+Stress.f, data=data.df)

>

> anova(lm.01)

Analysis of Variance Table

Response: pigment.2

|           | Df  | Sum Sq | Mean Sq | F value | Pr(>F)        |
|-----------|-----|--------|---------|---------|---------------|
| Age.f     | 2   | 0.0697 | 0.0349  | 2.2027  | 0.1126213     |
| sex       | 2   | 0.0030 | 0.0015  | 0.0950  | 0.9093979     |
| Temp.f    | 1   | 0.2188 | 0.2188  | 13.8224 | 0.0002474 *** |
| Diet.f    | 2   | 0.0420 | 0.0210  | 1.3280  | 0.2668521     |
| Stress.f  | 1   | 0.0052 | 0.0052  | 0.3262  | 0.5684291     |
| Residuals | 253 | 4.0043 | 0.0158  |         |               |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

> summary(lm.01)

Call:

lm(formula = pigment.2 ~ Age.f + sex + Temp.f + Diet.f + Stress.f,  
data = data.df)

Residuals:

| Min      | 1Q       | Median   | 3Q      | Max     |
|----------|----------|----------|---------|---------|
| -0.17592 | -0.05392 | -0.02286 | 0.03272 | 1.40830 |

Coefficients:

|                | Estimate   | Std. Error | t value | Pr(> t )     |
|----------------|------------|------------|---------|--------------|
| (Intercept)    | 0.2009343  | 0.0252973  | 7.943   | 6.49e-14 *** |
| Age.f 4 months | -0.0274633 | 0.0189327  | -1.451  | 0.148137     |

```

Age.f 7 months  0.0180812 0.0231164 0.782 0.434842
sexj          -0.0131070 0.0244298 -0.537 0.592074
sexm          -0.0001576 0.0177727 -0.009 0.992931
Temp.fWarm    0.0569463 0.0158236 3.599 0.000385 ***
Diet.f mixed diet -0.0268071 0.0210326 -1.275 0.203638
Diet.f phyto diet -0.0327208 0.0206237 -1.587 0.113860
Stress.f stress 0.0089549 0.0156797 0.571 0.568429

```

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 0.1258 on 253 degrees of freedom

Multiple R-squared: 0.07799, Adjusted R-squared: 0.04883

F-statistic: 2.675 on 8 and 253 DF, p-value: 0.007744

>

> # fit linear model with main effects + first-order interactions

>

```

> lm.02 <- lm(formula=pigment.2 ~ Age.f+sex+Temp.f+
+ Diet.f+Stress.f+Age.f:sex+Age.f:Temp.f+Age.f:Diet.f+Age.f:Stress.f+
+ sex:Temp.f+sex:Diet.f+sex:Stress.f+Temp.f:Diet.f+Temp.f:Stress.f+
+ Diet.f:Stress.f, data=data.df)

```

>

> anova(lm.02)

Analysis of Variance Table

Response: pigment.2

|                 | Df  | Sum Sq  | Mean Sq | F value | Pr(>F)        |
|-----------------|-----|---------|---------|---------|---------------|
| Age.f           | 2   | 0.06972 | 0.03486 | 2.5288  | 0.08198 .     |
| sex             | 2   | 0.00301 | 0.00150 | 0.1091  | 0.89671       |
| Temp.f          | 1   | 0.21877 | 0.21877 | 15.8691 | 9.123e-05 *** |
| Diet.f          | 2   | 0.04204 | 0.02102 | 1.5246  | 0.21991       |
| Stress.f        | 1   | 0.00516 | 0.00516 | 0.3745  | 0.54119       |
| Age.f:sex       | 3   | 0.06446 | 0.02149 | 1.5587  | 0.20022       |
| Age.f:Temp.f    | 2   | 0.12786 | 0.06393 | 4.6372  | 0.01061 *     |
| Age.f:Diet.f    | 4   | 0.11297 | 0.02824 | 2.0487  | 0.08846 .     |
| Age.f:Stress.f  | 2   | 0.02167 | 0.01083 | 0.7859  | 0.45695       |
| sex:Temp.f      | 2   | 0.03116 | 0.01558 | 1.1301  | 0.32480       |
| sex:Diet.f      | 4   | 0.08605 | 0.02151 | 1.5604  | 0.18577       |
| sex:Stress.f    | 2   | 0.05497 | 0.02749 | 1.9939  | 0.13852       |
| Temp.f:Diet.f   | 2   | 0.28292 | 0.14146 | 10.2612 | 5.397e-05 *** |
| Temp.f:Stress.f | 1   | 0.03309 | 0.03309 | 2.4006  | 0.12267       |
| Diet.f:Stress.f | 2   | 0.03216 | 0.01608 | 1.1664  | 0.31333       |
| Residuals       | 229 | 3.15697 | 0.01379 |         |               |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

> summary(lm.02)

Call:

```

lm(formula = pigment.2 ~ Age.f + sex + Temp.f + Diet.f + Stress.f +
  Age.f:sex + Age.f:Temp.f + Age.f:Diet.f + Age.f:Stress.f +

```

sex:Temp.f + sex:Diet.f + sex:Stress.f + Temp.f:Diet.f +  
Temp.f:Stress.f + Diet.f:Stress.f, data = data.df)

Residuals:

| Min       | 1Q        | Median    | 3Q       | Max      |
|-----------|-----------|-----------|----------|----------|
| -0.240283 | -0.051461 | -0.003652 | 0.033209 | 1.256785 |

Coefficients: (1 not defined because of singularities)

|                                   | Estimate   | Std. Error | t value | Pr(> t )     |
|-----------------------------------|------------|------------|---------|--------------|
| (Intercept)                       | 0.1795570  | 0.0464068  | 3.869   | 0.000142 *** |
| Age.f 4 months                    | 0.0089271  | 0.0500167  | 0.178   | 0.858502     |
| Age.f 7 months                    | -0.1053976 | 0.0741129  | -1.422  | 0.156351     |
| sexj                              | 0.0272880  | 0.0568414  | 0.480   | 0.631634     |
| sexm                              | -0.0112212 | 0.0466208  | -0.241  | 0.810010     |
| Temp.fWarm                        | 0.0997757  | 0.0465914  | 2.142   | 0.033289 *   |
| Diet.f mixed diet                 | 0.0180611  | 0.0527773  | 0.342   | 0.732505     |
| Diet.f phyto diet                 | 0.0485684  | 0.0518537  | 0.937   | 0.349929     |
| Stress.f stress                   | 0.0124009  | 0.0478182  | 0.259   | 0.795610     |
| Age.f 4 months:sexj               | 0.0440393  | 0.0577127  | 0.763   | 0.446202     |
| Age.f 7 months:sexj               | NA         | NA         | NA      | NA           |
| Age.f 4 months:sexm               | -0.0027528 | 0.0401643  | -0.069  | 0.945417     |
| Age.f 7 months:sexm               | 0.1008878  | 0.0451889  | 2.233   | 0.026544 *   |
| Age.f 4 months:Temp.fWarm         | 0.0154835  | 0.0364858  | 0.424   | 0.671694     |
| Age.f 7 months:Temp.fWarm         | 0.1219603  | 0.0519323  | 2.348   | 0.019703 *   |
| Age.f 4 months:Diet.f mixed diet  | -0.0861893 | 0.0475602  | -1.812  | 0.071263     |
| Age.f 7 months:Diet.f mixed diet  | 0.0462507  | 0.0734547  | 0.630   | 0.529552     |
| Age.f 4 months:Diet.f phyto diet  | -0.0211360 | 0.0456251  | -0.463  | 0.643622     |
| Age.f 7 months:Diet.f phyto diet  | 0.0281437  | 0.0755655  | 0.372   | 0.709909     |
| Age.f 4 months:Stress.f stress    | -0.0260932 | 0.0358783  | -0.727  | 0.467804     |
| Age.f 7 months:Stress.f stress    | -0.0501323 | 0.0512827  | -0.978  | 0.329320     |
| sexj:Temp.fWarm                   | -0.0827348 | 0.0485762  | -1.703  | 0.089888     |
| sexm:Temp.fWarm                   | 0.0045970  | 0.0345833  | 0.133   | 0.894369     |
| sexj:Diet.f mixed diet            | -0.0389640 | 0.0603553  | -0.646  | 0.519199     |
| sexm:Diet.f mixed diet            | -0.0240980 | 0.0471251  | -0.511  | 0.609590     |
| sexj:Diet.f phyto diet            | 0.0791676  | 0.0580776  | 1.363   | 0.174179     |
| sexm:Diet.f phyto diet            | -0.0154420 | 0.0466757  | -0.331  | 0.741071     |
| sexj:Stress.f stress              | -0.0769372 | 0.0466407  | -1.650  | 0.100402     |
| sexm:Stress.f stress              | 0.0006265  | 0.0346543  | 0.018   | 0.985592     |
| Temp.fWarm:Diet.f mixed diet      | -0.0639607 | 0.0414696  | -1.542  | 0.124369     |
| Temp.fWarm:Diet.f phyto diet      | -0.1635938 | 0.0401585  | -4.074  | 6.38e-05 *** |
| Temp.fWarm:Stress.f stress        | 0.0542630  | 0.0323168  | 1.679   | 0.094497     |
| Diet.f mixed diet:Stress.f stress | 0.0258995  | 0.0404464  | 0.640   | 0.522590     |
| Diet.f phyto diet:Stress.f stress | -0.0291106 | 0.0405285  | -0.718  | 0.473320     |

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 0.1174 on 229 degrees of freedom  
Multiple R-squared: 0.2731, Adjusted R-squared: 0.1715  
F-statistic: 2.688 on 32 and 229 DF, p-value: 1.153e-05

```

>
> # reduced model
>
> lm.03 <- lm(formula=pigment.2 ~ Age.f+Temp.f+
+   Diet.f+Age.f:Temp.f+Age.f:Diet.f+Temp.f:Diet.f, data=data.df)
>
> anova(lm.03)

```

# Analysis of Variance Table

Response: pigment.2

|               | Df  | Sum Sq | Mean Sq | F value | Pr(>F)        |
|---------------|-----|--------|---------|---------|---------------|
| Age.f         | 2   | 0.0697 | 0.0349  | 2.4612  | 0.0874172 .   |
| Temp.f        | 1   | 0.2191 | 0.2191  | 15.4709 | 0.0001088 *** |
| Diet.f        | 2   | 0.0393 | 0.0197  | 1.3884  | 0.2514093     |
| Age.f:Temp.f  | 2   | 0.1280 | 0.0640  | 4.5199  | 0.0118017 *   |
| Age.f:Diet.f  | 4   | 0.1034 | 0.0258  | 1.8241  | 0.1247132     |
| Temp.f:Diet.f | 2   | 0.2705 | 0.1352  | 9.5474  | 0.0001013 *** |
| Residuals     | 248 | 3.5129 | 0.0142  |         |               |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

```

> summary(lm.03)

```

Call:

```

lm(formula = pigment.2 ~ Age.f + Temp.f + Diet.f + Age.f:Temp.f +
  Age.f:Diet.f + Temp.f:Diet.f, data = data.df)

```

Residuals:

| Min      | 1Q       | Median   | 3Q      | Max     |
|----------|----------|----------|---------|---------|
| -0.19041 | -0.04942 | -0.01561 | 0.03647 | 1.30666 |

Coefficients:

|                                  | Estimate | Std. Error | t value | Pr(> t )     |
|----------------------------------|----------|------------|---------|--------------|
| (Intercept)                      | 0.16897  | 0.02470    | 6.842   | 6.09e-11 *** |
| Age.f 4 months                   | 0.01318  | 0.03673    | 0.359   | 0.720011     |
| Age.f 7 months                   | -0.06814 | 0.06579    | -1.036  | 0.301340     |
| Temp.fWarm                       | 0.08459  | 0.03199    | 2.644   | 0.008709 **  |
| Diet.f mixed diet                | 0.01677  | 0.03319    | 0.505   | 0.613917     |
| Diet.f phyto diet                | 0.06703  | 0.03296    | 2.034   | 0.043024 *   |
| Age.f 4 months:Temp.fWarm        | 0.04239  | 0.03385    | 1.252   | 0.211647     |
| Age.f 7 months:Temp.fWarm        | 0.13836  | 0.04161    | 3.325   | 0.001017 **  |
| Age.f 4 months:Diet.f mixed diet | -0.08918 | 0.04382    | -2.035  | 0.042901 *   |
| Age.f 7 months:Diet.f mixed diet | 0.04865  | 0.06899    | 0.705   | 0.481358     |
| Age.f 4 months:Diet.f phyto diet | -0.06480 | 0.04208    | -1.540  | 0.124822     |
| Age.f 7 months:Diet.f phyto diet | 0.00111  | 0.07100    | 0.016   | 0.987535     |
| Temp.fWarm:Diet.f mixed diet     | -0.02959 | 0.03967    | -0.746  | 0.456342     |
| Temp.fWarm:Diet.f phyto diet     | -0.15054 | 0.03888    | -3.872  | 0.000138 *** |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 0.119 on 248 degrees of freedom



Multiple R-squared: 0.1911, Adjusted R-squared: 0.1487  
F-statistic: 4.508 on 13 and 248 DF, p-value: 6.7e-07

```
>
> # analyse pigment.3
>
> # fit linear model with main effects only
>
> lm.01 <- lm(formula=pigment.3 ~ Age.f+sex+Temp.f+
+ Diet.f+Stress.f, data=data.df)
>
> anova(lm.01)
Analysis of Variance Table
```

Response: pigment.3

|           | Df  | Sum Sq  | Mean Sq | F value | Pr(>F)        |
|-----------|-----|---------|---------|---------|---------------|
| Age.f     | 2   | 0.08392 | 0.04196 | 17.0167 | 1.165e-07 *** |
| sex       | 2   | 0.00243 | 0.00121 | 0.4926  | 0.61165       |
| Temp.f    | 1   | 0.00807 | 0.00807 | 3.2738  | 0.07158 .     |
| Diet.f    | 2   | 0.00955 | 0.00477 | 1.9358  | 0.14644       |
| Stress.f  | 1   | 0.00276 | 0.00276 | 1.1182  | 0.29130       |
| Residuals | 253 | 0.62382 | 0.00247 |         |               |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

```
> summary(lm.01)
```

Call:

```
lm(formula = pigment.3 ~ Age.f + sex + Temp.f + Diet.f + Stress.f,
    data = data.df)
```

Residuals:

| Min      | 1Q       | Median   | 3Q      | Max     |
|----------|----------|----------|---------|---------|
| -0.10248 | -0.03159 | -0.01067 | 0.02356 | 0.22713 |

Coefficients:

|                   | Estimate  | Std. Error | t value | Pr(> t )    |
|-------------------|-----------|------------|---------|-------------|
| (Intercept)       | 0.135071  | 0.009985   | 13.528  | < 2e-16 *** |
| Age.f 4 months    | -0.024764 | 0.007473   | -3.314  | 0.00105 **  |
| Age.f 7 months    | 0.026396  | 0.009124   | 2.893   | 0.00415 **  |
| sexj              | -0.010536 | 0.009642   | -1.093  | 0.27558     |
| sexm              | -0.004537 | 0.007015   | -0.647  | 0.51841     |
| Temp.fWarm        | -0.011341 | 0.006246   | -1.816  | 0.07059 .   |
| Diet.f mixed diet | -0.004452 | 0.008302   | -0.536  | 0.59227     |
| Diet.f phyto diet | 0.009096  | 0.008140   | 1.117   | 0.26488     |
| Stress.f stress   | -0.006544 | 0.006189   | -1.057  | 0.29130     |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 0.04966 on 253 degrees of freedom  
Multiple R-squared: 0.1461, Adjusted R-squared: 0.1191

F-statistic: 5.41 on 8 and 253 DF, p-value: 2.675e-06

```
>
> # fit linear model with main effects + first-order interactions
>
> lm.02 <- lm(formula=pigment.3 ~ Age.f+sex+Temp.f+
+ Diet.f+Stress.f+Age.f:sex+Age.f:Temp.f+Age.f:Diet.f+Age.f:Stress.f+
+ sex:Temp.f+sex:Diet.f+sex:Stress.f+Temp.f:Diet.f+Temp.f:Stress.f+
+ Diet.f:Stress.f, data=data.df)
>
> anova(lm.02)
Analysis of Variance Table
```

Response: pigment.3

|                 | Df  | Sum Sq  | Mean Sq | F value | Pr(>F)        |
|-----------------|-----|---------|---------|---------|---------------|
| Age.f           | 2   | 0.08392 | 0.04196 | 24.2526 | 2.797e-10 *** |
| sex             | 2   | 0.00243 | 0.00121 | 0.7020  | 0.49666       |
| Temp.f          | 1   | 0.00807 | 0.00807 | 4.6659  | 0.03181 *     |
| Diet.f          | 2   | 0.00955 | 0.00477 | 2.7589  | 0.06547 .     |
| Stress.f        | 1   | 0.00276 | 0.00276 | 1.5938  | 0.20807       |
| Age.f:sex       | 3   | 0.00553 | 0.00184 | 1.0648  | 0.36480       |
| Age.f:Temp.f    | 2   | 0.07869 | 0.03935 | 22.7425 | 9.790e-10 *** |
| Age.f:Diet.f    | 4   | 0.02074 | 0.00519 | 2.9971  | 0.01943 *     |
| Age.f:Stress.f  | 2   | 0.00535 | 0.00267 | 1.5452  | 0.21549       |
| sex:Temp.f      | 2   | 0.00275 | 0.00137 | 0.7938  | 0.45335       |
| sex:Diet.f      | 4   | 0.00592 | 0.00148 | 0.8552  | 0.49168       |
| sex:Stress.f    | 2   | 0.00483 | 0.00242 | 1.3964  | 0.24958       |
| Temp.f:Diet.f   | 2   | 0.08589 | 0.04294 | 24.8220 | 1.750e-10 *** |
| Temp.f:Stress.f | 1   | 0.00098 | 0.00098 | 0.5665  | 0.45241       |
| Diet.f:Stress.f | 2   | 0.01697 | 0.00849 | 4.9058  | 0.00820 **    |
| Residuals       | 229 | 0.39618 | 0.00173 |         |               |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

```
> summary(lm.02)
```

Call:

```
lm(formula = pigment.3 ~ Age.f + sex + Temp.f + Diet.f + Stress.f +
  Age.f:sex + Age.f:Temp.f + Age.f:Diet.f + Age.f:Stress.f +
  sex:Temp.f + sex:Diet.f + sex:Stress.f + Temp.f:Diet.f +
  Temp.f:Stress.f + Diet.f:Stress.f, data = data.df)
```

Residuals:

| Min       | 1Q        | Median    | 3Q       | Max      |
|-----------|-----------|-----------|----------|----------|
| -0.130270 | -0.022784 | -0.002525 | 0.018496 | 0.176906 |

Coefficients: (1 not defined because of singularities)

|                | Estimate   | Std. Error | t value | Pr(> t )     |
|----------------|------------|------------|---------|--------------|
| (Intercept)    | 0.0921986  | 0.0164396  | 5.608   | 5.86e-08 *** |
| Age.f 4 months | -0.0106730 | 0.0177184  | -0.602  | 0.54752      |
| Age.f 7 months | 0.0001295  | 0.0262545  | 0.005   | 0.99607      |

|                                   |            |           |        |              |
|-----------------------------------|------------|-----------|--------|--------------|
| sexj                              | -0.0029784 | 0.0201361 | -0.148 | 0.88254      |
| sexm                              | 0.0065108  | 0.0165154 | 0.394  | 0.69378      |
| Temp.fWarm                        | 0.0764138  | 0.0165050 | 4.630  | 6.14e-06 *** |
| Diet.f mixed diet                 | 0.0164381  | 0.0186964 | 0.879  | 0.38021      |
| Diet.f phyto diet                 | 0.0530219  | 0.0183692 | 2.886  | 0.00427 **   |
| Stress.f stress                   | 0.0082727  | 0.0169396 | 0.488  | 0.62576      |
| Age.f 4 months:sexj               | 0.0246818  | 0.0204447 | 1.207  | 0.22858      |
| Age.f 7 months:sexj               | NA         | NA        | NA     | NA           |
| Age.f 4 months:sexm               | 0.0055115  | 0.0142282 | 0.387  | 0.69885      |
| Age.f 7 months:sexm               | 0.0180206  | 0.0160082 | 1.126  | 0.26146      |
| Age.f 4 months:Temp.fWarm         | -0.0009582 | 0.0129251 | -0.074 | 0.94096      |
| Age.f 7 months:Temp.fWarm         | -0.1067648 | 0.0183970 | -5.803 | 2.15e-08 *** |
| Age.f 4 months:Diet.f mixed diet  | -0.0263869 | 0.0168482 | -1.566 | 0.11869      |
| Age.f 7 months:Diet.f mixed diet  | 0.0275563  | 0.0260213 | 1.059  | 0.29072      |
| Age.f 4 months:Diet.f phyto diet  | -0.0154569 | 0.0161627 | -0.956 | 0.33991      |
| Age.f 7 months:Diet.f phyto diet  | 0.0685654  | 0.0267691 | 2.561  | 0.01107 *    |
| Age.f 4 months:Stress.f stress    | -0.0084564 | 0.0127099 | -0.665 | 0.50650      |
| Age.f 7 months:Stress.f stress    | 0.0249602  | 0.0181669 | 1.374  | 0.17080      |
| sexj:Temp.fWarm                   | -0.0291598 | 0.0172081 | -1.695 | 0.09152 .    |
| sexm:Temp.fWarm                   | -0.0140494 | 0.0122511 | -1.147 | 0.25267      |
| sexj:Diet.f mixed diet            | -0.0076149 | 0.0213808 | -0.356 | 0.72205      |
| sexm:Diet.f mixed diet            | -0.0047131 | 0.0166941 | -0.282 | 0.77795      |
| sexj:Diet.f phyto diet            | 0.0261263  | 0.0205740 | 1.270  | 0.20542      |
| sexm:Diet.f phyto diet            | -0.0064206 | 0.0165349 | -0.388 | 0.69815      |
| sexj:Stress.f stress              | -0.0236672 | 0.0165225 | -1.432 | 0.15339      |
| sexm:Stress.f stress              | -0.0123996 | 0.0122763 | -1.010 | 0.31354      |
| Temp.fWarm:Diet.f mixed diet      | -0.0624940 | 0.0146906 | -4.254 | 3.06e-05 *** |
| Temp.fWarm:Diet.f phyto diet      | -0.0926046 | 0.0142262 | -6.509 | 4.71e-10 *** |
| Temp.fWarm:Stress.f stress        | -0.0047806 | 0.0114482 | -0.418 | 0.67664      |
| Diet.f mixed diet:Stress.f stress | 0.0311358  | 0.0143281 | 2.173  | 0.03080 *    |
| Diet.f phyto diet:Stress.f stress | -0.0069669 | 0.0143572 | -0.485 | 0.62796      |

---  
Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 0.04159 on 229 degrees of freedom  
Multiple R-squared: 0.4577, Adjusted R-squared: 0.3819  
F-statistic: 6.04 on 32 and 229 DF, p-value: < 2.2e-16

```
>
> # reduced model
>
> lm.03 <- lm(formula=pigment.3 ~ Age.f+Temp.f+
+ Diet.f+Age.f:Temp.f+Age.f:Diet.f+Temp.f:Diet.f, data=data.df)
>
> # add 3 factor interaction
>
> lm.03 <- lm(formula=pigment.3 ~ Age.f+Temp.f+
+ Diet.f+Age.f:Temp.f+Age.f:Diet.f+Temp.f:Diet.f+Age.f:Temp.f:Diet.f,
+ data=data.df)
>
```

```
>
> anova(lm.03)
Analysis of Variance Table
```

```
Response: pigment.3
```

|                     | Df  | Sum Sq  | Mean Sq | F value | Pr(>F)        |
|---------------------|-----|---------|---------|---------|---------------|
| Age.f               | 2   | 0.08392 | 0.04196 | 23.0476 | 6.786e-10 *** |
| Temp.f              | 1   | 0.00789 | 0.00789 | 4.3346  | 0.03839 *     |
| Diet.f              | 2   | 0.00897 | 0.00448 | 2.4628  | 0.08731 .     |
| Age.f:Temp.f        | 2   | 0.08163 | 0.04081 | 22.4196 | 1.152e-09 *** |
| Age.f:Diet.f        | 4   | 0.02108 | 0.00527 | 2.8946  | 0.02282 *     |
| Temp.f:Diet.f       | 2   | 0.06486 | 0.03243 | 17.8137 | 6.009e-08 *** |
| Age.f:Temp.f:Diet.f | 4   | 0.01800 | 0.00450 | 2.4719  | 0.04519 *     |
| Residuals           | 244 | 0.44420 | 0.00182 |         |               |

```
---
```

```
Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
> summary(lm.03)
```

```
Call:
```

```
lm(formula = pigment.3 ~ Age.f + Temp.f + Diet.f + Age.f:Temp.f +
    Age.f:Diet.f + Temp.f:Diet.f + Age.f:Temp.f:Diet.f, data = data.df)
```

```
Residuals:
```

| Min       | 1Q        | Median    | 3Q       | Max      |
|-----------|-----------|-----------|----------|----------|
| -0.142802 | -0.021842 | -0.003352 | 0.019563 | 0.201492 |

```
Coefficients:
```

|                                             | Estimate  | Std. Error | t value | Pr(> t )    |
|---------------------------------------------|-----------|------------|---------|-------------|
| (Intercept)                                 | 0.091752  | 0.009541   | 9.617   | < 2e-16 *** |
| Age.f 4 months                              | 0.007194  | 0.016525   | 0.435   |             |
| Age.f 7 months                              | -0.035689 | 0.031643   | -1.128  |             |
| Temp.fWarm                                  | 0.046903  | 0.013493   | 3.476   |             |
| Diet.f mixed diet                           | 0.025319  | 0.013493   | 1.877   |             |
| Diet.f phyto diet                           | 0.056323  | 0.013493   | 4.174   |             |
| Age.f 4 months:Temp.fWarm                   | -0.011046 | 0.023370   | -0.473  |             |
| Age.f 7 months:Temp.fWarm                   | 0.039816  | 0.044750   | 0.890   |             |
| Age.f 4 months:Diet.f mixed diet            | -0.038488 | 0.021334   | -1.804  |             |
| Age.f 7 months:Diet.f mixed diet            | 0.094724  | 0.034399   | 2.754   |             |
| Age.f 4 months:Diet.f phyto diet            | -0.043220 | 0.021334   | -2.026  |             |
| Age.f 7 months:Diet.f phyto diet            | 0.114061  | 0.035698   | 3.195   |             |
| Temp.fWarm:Diet.f mixed diet                | -0.039311 | 0.019081   | -2.060  |             |
| Temp.fWarm:Diet.f phyto diet                | -0.083654 | 0.019081   | -4.384  |             |
| Age.f 4 months:Temp.fWarm:Diet.f mixed diet | 0.015931  | 0.031643   | 0.503   |             |
| Age.f 7 months:Temp.fWarm:Diet.f mixed diet | -0.130453 | 0.049575   | -2.631  |             |
| Age.f 4 months:Temp.fWarm:Diet.f phyto diet | 0.029647  | 0.030170   | 0.983   |             |
| Age.f 7 months:Temp.fWarm:Diet.f phyto diet | -0.130109 | 0.050933   | -2.555  |             |

|                                             |              |
|---------------------------------------------|--------------|
| Temp.fWarm                                  | 0.000602 *** |
| Diet.f mixed diet                           | 0.061778 .   |
| Diet.f phyto diet                           | 4.16e-05 *** |
| Age.f 4 months:Temp.fWarm                   | 0.636891     |
| Age.f 7 months:Temp.fWarm                   | 0.374472     |
| Age.f 4 months:Diet.f mixed diet            | 0.072446 .   |
| Age.f 7 months:Diet.f mixed diet            | 0.006337 **  |
| Age.f 4 months:Diet.f phyto diet            | 0.043863 *   |
| Age.f 7 months:Diet.f phyto diet            | 0.001582 **  |
| Temp.fWarm:Diet.f mixed diet                | 0.040443 *   |
| Temp.fWarm:Diet.f phyto diet                | 1.73e-05 *** |
| Age.f 4 months:Temp.fWarm:Diet.f mixed diet | 0.615092     |
| Age.f 7 months:Temp.fWarm:Diet.f mixed diet | 0.009044 **  |
| Age.f 4 months:Temp.fWarm:Diet.f phyto diet | 0.326753     |
| Age.f 7 months:Temp.fWarm:Diet.f phyto diet | 0.011242 *   |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 0.04267 on 244 degrees of freedom  
Multiple R-squared: 0.392, Adjusted R-squared: 0.3496  
F-statistic: 9.252 on 17 and 244 DF, p-value: < 2.2e-16

>

> # analyse CL

>

> # fit linear model with main effects only

>

> lm.01 <- lm(formula=CL ~ Age.f+sex+Temp.f+  
+ Diet.f+Stress.f, data=data.df)

>

> anova(lm.01)

Analysis of Variance Table

Response: CL

|           | Df  | Sum Sq  | Mean Sq | F value | Pr(>F)      |
|-----------|-----|---------|---------|---------|-------------|
| Age.f     | 2   | 6.313   | 3.156   | 4.3713  | 0.013603 *  |
| sex       | 2   | 6.991   | 3.495   | 4.8409  | 0.008646 ** |
| Temp.f    | 1   | 4.259   | 4.259   | 5.8989  | 0.015848 *  |
| Diet.f    | 2   | 4.618   | 2.309   | 3.1979  | 0.042504 *  |
| Stress.f  | 1   | 0.071   | 0.071   | 0.0987  | 0.753674    |
| Residuals | 253 | 182.678 | 0.722   |         |             |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

> summary(lm.01)

Call:

lm(formula = CL ~ Age.f + sex + Temp.f + Diet.f + Stress.f, data = data.df)

Residuals:

|     |    |        |    |     |
|-----|----|--------|----|-----|
| Min | 1Q | Median | 3Q | Max |
|-----|----|--------|----|-----|

-1.96660 -0.55706 -0.02328' 0.52822 2.57377

Coefficients:

|                   | Estimate  | Std. Error | t value | Pr(> t )     |
|-------------------|-----------|------------|---------|--------------|
| (Intercept)       | 9.466755  | 0.170866   | 55.405  | < 2e-16 ***  |
| Age.f 4 months    | -0.493420 | 0.127877   | -3.859  | 0.000145 *** |
| Age.f 7 months    | -0.365479 | 0.156136   | -2.341  | 0.020021 *   |
| sexj              | -0.419730 | 0.165006   | -2.544  | 0.011564 *   |
| sexm              | 0.001816  | 0.120043   | 0.015   | 0.987941     |
| Temp.fWarm        | 0.255877  | 0.106878   | 2.394   | 0.017390 *   |
| Diet.f mixed diet | 0.173965  | 0.142061   | 1.225   | 0.221870     |
| Diet.f phyto diet | 0.347044  | 0.139299   | 2.491   | 0.013367 *   |
| Stress.f stress   | -0.033269 | 0.105905   | -0.314  | 0.753674     |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 0.8497 on 253 degrees of freedom

Multiple R-squared: 0.1086, Adjusted R-squared: 0.0804

F-statistic: 3.852 on 8 and 253 DF, p-value: 0.0002674

>

> # fit linear model with main effects + first-order interactions

>

```
> lm.02 <- lm(formula=CL ~ Age.f+sex+Temp.f+
+ Diet.f+Stress.f+Age.f:sex+Age.f:Temp.f+Age.f:Diet.f+Age.f:Stress.f+
+ sex:Temp.f+sex:Diet.f+sex:Stress.f+Temp.f:Diet.f+Temp.f:Stress.f+
+ Diet.f:Stress.f, data=data.df)
```

>

> anova(lm.02)

Analysis of Variance Table

Response: CL

|                 | Df  | Sum Sq  | Mean Sq | F value | Pr(>F)      |
|-----------------|-----|---------|---------|---------|-------------|
| Age.f           | 2   | 6.313   | 3.156   | 4.5434  | 0.011614 *  |
| sex             | 2   | 6.991   | 3.495   | 5.0315  | 0.007269 ** |
| Temp.f          | 1   | 4.259   | 4.259   | 6.1311  | 0.014007 *  |
| Diet.f          | 2   | 4.618   | 2.309   | 3.3238  | 0.037762 *  |
| Stress.f        | 1   | 0.071   | 0.071   | 0.1026  | 0.749061    |
| Age.f:sex       | 3   | 3.416   | 1.139   | 1.6389  | 0.181169    |
| Age.f:Temp.f    | 2   | 2.961   | 1.481   | 2.1314  | 0.121023    |
| Age.f:Diet.f    | 4   | 10.131  | 2.533   | 3.6459  | 0.006685 ** |
| Age.f:Stress.f  | 2   | 0.267   | 0.134   | 0.1923  | 0.825156    |
| sex:Temp.f      | 2   | 0.110   | 0.055   | 0.0791  | 0.923966    |
| sex:Diet.f      | 4   | 2.504   | 0.626   | 0.9013  | 0.463943    |
| sex:Stress.f    | 2   | 2.700   | 1.350   | 1.9433  | 0.145588    |
| Temp.f:Diet.f   | 2   | 0.169   | 0.084   | 0.1213  | 0.885818    |
| Temp.f:Stress.f | 1   | 0.294   | 0.294   | 0.4238  | 0.515686    |
| Diet.f:Stress.f | 2   | 1.038   | 0.519   | 0.7473  | 0.474799    |
| Residuals       | 229 | 159.087 | 0.695   |         |             |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1  
> summary(lm.02)

Call:

```
lm(formula = CL ~ Age.f + sex + Temp.f + Diet.f + Stress.f +
    Age.f:sex + Age.f:Temp.f + Age.f:Diet.f + Age.f:Stress.f +
    sex:Temp.f + sex:Diet.f + sex:Stress.f + Temp.f:Diet.f +
    Temp.f:Stress.f + Diet.f:Stress.f, data = data.df)
```

Residuals:

| Min       | 1Q        | Median   | 3Q       | Max      |
|-----------|-----------|----------|----------|----------|
| -2.247189 | -0.484581 | 0.008284 | 0.573951 | 2.212888 |

Coefficients: (1 not defined because of singularities)

|                                   | Estimate | Std. Error | t value | Pr(> t )     |
|-----------------------------------|----------|------------|---------|--------------|
| (Intercept)                       | 9.86057  | 0.32943    | 29.932  | < 2e-16 ***  |
| Age.f 4 months                    | -1.42877 | 0.35506    | -4.024  | 7.78e-05 *** |
| Age.f 7 months                    | -1.24928 | 0.52611    | -2.375  | 0.01839 *    |
| sexj                              | -0.57920 | 0.40350    | -1.435  | 0.15253      |
| sexm                              | -0.18960 | 0.33095    | -0.573  | 0.56727      |
| Temp.fWarm                        | 0.08552  | 0.33074    | 0.259   | 0.79619      |
| Diet.f mixed diet                 | 0.21033  | 0.37465    | 0.561   | 0.57508      |
| Diet.f phyto diet                 | -0.04382 | 0.36810    | -0.119  | 0.90535      |
| Stress.f stress                   | -0.20366 | 0.33945    | -0.600  | 0.54911      |
| Age.f 4 months:sexj               | 0.01892  | 0.40969    | 0.046   | 0.96321      |
| Age.f 7 months:sexj               | NA       | NA         | NA      | NA           |
| Age.f 4 months:sexm               | 0.31666  | 0.28512    | 1.111   | 0.26789      |
| Age.f 7 months:sexm               | 0.64270  | 0.32078    | 2.004   | 0.04630 *    |
| Age.f 4 months:Temp.fWarm         | 0.50228  | 0.25900    | 1.939   | 0.05370 .    |
| Age.f 7 months:Temp.fWarm         | 0.05510  | 0.36865    | 0.149   | 0.88131      |
| Age.f 4 months:Diet.f mixed diet  | 0.41246  | 0.33762    | 1.222   | 0.22309      |
| Age.f 7 months:Diet.f mixed diet  | 0.22317  | 0.52144    | 0.428   | 0.66906      |
| Age.f 4 months:Diet.f phyto diet  | 0.87333  | 0.32388    | 2.696   | 0.00753 **   |
| Age.f 7 months:Diet.f phyto diet  | 0.99523  | 0.53642    | 1.855   | 0.06484 .    |
| Age.f 4 months:Stress.f stress    | 0.09203  | 0.25469    | 0.361   | 0.71819      |
| Age.f 7 months:Stress.f stress    | 0.12372  | 0.36404    | 0.340   | 0.73429      |
| sexj:Temp.fWarm                   | -0.02082 | 0.34483    | -0.060  | 0.95191      |
| sexm:Temp.fWarm                   | -0.11780 | 0.24550    | -0.480  | 0.63178      |
| sexj:Diet.f mixed diet            | -0.68752 | 0.42845    | -1.605  | 0.10994      |
| sexm:Diet.f mixed diet            | -0.19242 | 0.33453    | -0.575  | 0.56573      |
| sexj:Diet.f phyto diet            | -0.13344 | 0.41228    | -0.324  | 0.74649      |
| sexm:Diet.f phyto diet            | 0.15814  | 0.33134    | 0.477   | 0.63363      |
| sexj:Stress.f stress              | 0.62017  | 0.33109    | 1.873   | 0.06233 .    |
| sexm:Stress.f stress              | 0.04404  | 0.24600    | 0.179   | 0.85809      |
| Temp.fWarm:Diet.f mixed diet      | 0.12566  | 0.29438    | 0.427   | 0.66989      |
| Temp.fWarm:Diet.f phyto diet      | 0.10942  | 0.28508    | 0.384   | 0.70147      |
| Temp.fWarm:Stress.f stress        | -0.12133 | 0.22941    | -0.529  | 0.59740      |
| Diet.f mixed diet:Stress.f stress | 0.17982  | 0.28712    | 0.626   | 0.53174      |
| Diet.f phyto diet:Stress.f stress | -0.13188 | 0.28770    | -0.458  | 0.64709      |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 0.8335 on 229 degrees of freedom

Multiple R-squared: 0.2237, Adjusted R-squared: 0.1152

F-statistic: 2.062 on 32 and 229 DF, p-value: 0.001251

```
>
> # reduced model
>
> lm.03 <- lm(formula=CL ~ Age.f+Temp.f+
+ Diet.f+Age.f:Temp.f+Age.f:Diet.f+Temp.f:Diet.f, data=data.df)
>
> anova(lm.03)
Analysis of Variance Table
```

Response: CL

|               | Df  | Sum Sq  | Mean Sq | F value | Pr(>F)      |
|---------------|-----|---------|---------|---------|-------------|
| Age.f         | 2   | 6.313   | 3.156   | 4.4822  | 0.012239 *  |
| Temp.f        | 1   | 4.283   | 4.283   | 6.0817  | 0.014337 *  |
| Diet.f        | 2   | 5.558   | 2.779   | 3.9465  | 0.020547 *  |
| Age.f:Temp.f  | 2   | 3.611   | 1.806   | 2.5641  | 0.079027 .  |
| Age.f:Diet.f  | 4   | 9.937   | 2.484   | 3.5278  | 0.008033 ** |
| Temp.f:Diet.f | 2   | 0.592   | 0.296   | 0.4202  | 0.657382    |
| Residuals     | 248 | 174.637 | 0.704   |         |             |

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

```
> summary(lm.03)
```

Call:

```
lm(formula = CL ~ Age.f + Temp.f + Diet.f + Age.f:Temp.f + Age.f:Diet.f +
    Temp.f:Diet.f, data = data.df)
```

Residuals:

| Min       | 1Q        | Median   | 3Q       | Max      |
|-----------|-----------|----------|----------|----------|
| -2.280258 | -0.559862 | 0.000475 | 0.578919 | 2.630517 |

Coefficients:

|                                  | Estimate | Std. Error | t value | Pr(> t )     |
|----------------------------------|----------|------------|---------|--------------|
| (Intercept)                      | 9.61980  | 0.17413    | 55.244  | < 2e-16 ***  |
| Age.f 4 months                   | -1.18949 | 0.25894    | -4.594  | 6.93e-06 *** |
| Age.f 7 months                   | -0.72415 | 0.46386    | -1.561  | 0.119768     |
| Temp.fWarm                       | -0.03178 | 0.22555    | -0.141  | 0.888067     |
| Diet.f mixed diet                | -0.04762 | 0.23402    | -0.204  | 0.838908     |
| Diet.f phyto diet                | -0.13497 | 0.23237    | -0.581  | 0.561866     |
| Age.f 4 months:Temp.fWarm        | 0.50544  | 0.23865    | 2.118   | 0.035177 *   |
| Age.f 7 months:Temp.fWarm        | -0.06006 | 0.29338    | -0.205  | 0.837967     |
| Age.f 4 months:Diet.f mixed diet | 0.58182  | 0.30895    | 1.883   | 0.060842 .   |
| Age.f 7 months:Diet.f mixed diet | 0.37011  | 0.48645    | 0.761   | 0.447477     |
| Age.f 4 months:Diet.f phyto diet | 1.00151  | 0.29669    | 3.376   | 0.000855 *** |
| Age.f 7 months:Diet.f phyto diet | 0.98811  | 0.50062    | 1.974   | 0.049517 *   |



```
Temp.fWarm:Diet.f mixed diet    0.24526  0.27969  0.877 0.381390
Temp.fWarm:Diet.f phyto diet    0.08795  0.27412  0.321 0.748594
```

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 0.8392 on 248 degrees of freedom  
Multiple R-squared: 0.1478, Adjusted R-squared: 0.1032  
F-statistic: 3.309 on 13 and 248 DF, p-value: 0.0001134

```
>
> # analyse wetwteyes
>
> # fit linear model with main effects only
>
> lm.01 <- lm(formula=wetwteyes ~ Age.f+sex+Temp.f+
+   Diet.f+Stress.f, data=data.df)
>
> anova(lm.01)
Analysis of Variance Table
```

Response: wetwteyes

|           | Df  | Sum Sq    | Mean Sq   | F value | Pr(>F) |
|-----------|-----|-----------|-----------|---------|--------|
| Age.f     | 2   | 0.0001626 | 0.0000813 | 0.6720  | 0.5116 |
| sex       | 2   | 0.0000444 | 0.0000222 | 0.1837  | 0.8323 |
| Temp.f    | 1   | 0.0000828 | 0.0000828 | 0.6846  | 0.4088 |
| Diet.f    | 2   | 0.0001795 | 0.0000897 | 0.7418  | 0.4773 |
| Stress.f  | 1   | 0.0001311 | 0.0001311 | 1.0839  | 0.2988 |
| Residuals | 253 | 0.0306067 | 0.0001210 |         |        |

```
> summary(lm.01)
```

Call:

```
lm(formula = wetwteyes ~ Age.f + sex + Temp.f + Diet.f + Stress.f,
    data = data.df)
```

Residuals:

| Min        | 1Q         | Median     | 3Q        | Max       |
|------------|------------|------------|-----------|-----------|
| -0.0062549 | -0.0030521 | -0.0016300 | 0.0003135 | 0.1333925 |

Coefficients:

|                   | Estimate   | Std. Error | t value | Pr(> t )   |
|-------------------|------------|------------|---------|------------|
| (Intercept)       | 0.0071348  | 0.0022117  | 3.226   | 0.00142 ** |
| Age.f 4 months    | -0.0015674 | 0.0016552  | -0.947  | 0.34458    |
| Age.f 7 months    | -0.0004769 | 0.0020210  | -0.236  | 0.81366    |
| sexj              | 0.0001929  | 0.0021358  | 0.090   | 0.92811    |
| sexm              | 0.0008163  | 0.0015538  | 0.525   | 0.59982    |
| Temp.fWarm        | 0.0008526  | 0.0013834  | 0.616   | 0.53826    |
| Diet.f mixed diet | -0.0022368 | 0.0018388  | -1.216  | 0.22494    |
| Diet.f phyto diet | -0.0008948 | 0.0018031  | -0.496  | 0.62014    |
| Stress.f stress   | 0.0014271  | 0.0013708  | 1.041   | 0.29883    |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 0.011 on 253 degrees of freedom

Multiple R-squared: 0.01924, Adjusted R-squared: -0.01177

F-statistic: 0.6204 on 8 and 253 DF, p-value: 0.7604

>

> # fit linear model with main effects + first-order interactions

>

```
> lm.02 <- lm(formula=wetwteyes ~ Age.f+sex+Temp.f+
+ Diet.f+Stress.f+Age.f:sex+Age.f:Temp.f+Age.f:Diet.f+Age.f:Stress.f+
+ sex:Temp.f+sex:Diet.f+sex:Stress.f+Temp.f:Diet.f+Temp.f:Stress.f+
+ Diet.f:Stress.f, data=data.df)
```

>

> anova(lm.02)

Analysis of Variance Table

Response: wetwteyes

|                 | Df  | Sum Sq    | Mean Sq   | F value | Pr(>F) |
|-----------------|-----|-----------|-----------|---------|--------|
| Age.f           | 2   | 0.0001626 | 0.0000813 | 0.6607  | 0.5175 |
| sex             | 2   | 0.0000444 | 0.0000222 | 0.1806  | 0.8349 |
| Temp.f          | 1   | 0.0000828 | 0.0000828 | 0.6731  | 0.4128 |
| Diet.f          | 2   | 0.0001795 | 0.0000897 | 0.7293  | 0.4833 |
| Stress.f        | 1   | 0.0001311 | 0.0001311 | 1.0656  | 0.3030 |
| Age.f:sex       | 3   | 0.0000461 | 0.0000154 | 0.1250  | 0.9452 |
| Age.f:Temp.f    | 2   | 0.0003442 | 0.0001721 | 1.3987  | 0.2490 |
| Age.f:Diet.f    | 4   | 0.0003621 | 0.0000905 | 0.7357  | 0.5684 |
| Age.f:Stress.f  | 2   | 0.0004207 | 0.0002103 | 1.7095  | 0.1833 |
| sex:Temp.f      | 2   | 0.0001055 | 0.0000527 | 0.4287  | 0.6519 |
| sex:Diet.f      | 4   | 0.0003347 | 0.0000837 | 0.6800  | 0.6065 |
| sex:Stress.f    | 2   | 0.0000272 | 0.0000136 | 0.1105  | 0.8954 |
| Temp.f:Diet.f   | 2   | 0.0004739 | 0.0002369 | 1.9257  | 0.1481 |
| Temp.f:Stress.f | 1   | 0.0002577 | 0.0002577 | 2.0946  | 0.1492 |
| Diet.f:Stress.f | 2   | 0.0000575 | 0.0000287 | 0.2336  | 0.7918 |
| Residuals       | 229 | 0.0281771 | 0.0001230 |         |        |

> summary(lm.02)

Call:

```
lm(formula = wetwteyes ~ Age.f + sex + Temp.f + Diet.f + Stress.f +
    Age.f:sex + Age.f:Temp.f + Age.f:Diet.f + Age.f:Stress.f +
    sex:Temp.f + sex:Diet.f + sex:Stress.f + Temp.f:Diet.f +
    Temp.f:Stress.f + Diet.f:Stress.f, data = data.df)
```

Residuals:

| Min        | 1Q         | Median     | 3Q        | Max       |
|------------|------------|------------|-----------|-----------|
| -0.0123346 | -0.0027207 | -0.0006922 | 0.0012496 | 0.1252654 |

Coefficients: (1 not defined because of singularities)

|             | Estimate  | Std. Error | t value | Pr(> t ) |
|-------------|-----------|------------|---------|----------|
| (Intercept) | 0.0057090 | 0.0043842  | 1.302   | 0.194    |

|                                   |            |           |        |       |
|-----------------------------------|------------|-----------|--------|-------|
| Age.f 4 months                    | 0.0004327  | 0.0047253 | 0.092  | 0.927 |
| Age.f 7 months                    | 0.0030545  | 0.0070018 | 0.436  | 0.663 |
| sexj                              | 0.0006779  | 0.0053700 | 0.126  | 0.900 |
| sexm                              | -0.0001335 | 0.0044045 | -0.030 | 0.976 |
| Temp.fWarm                        | 0.0001658  | 0.0044017 | 0.038  | 0.970 |
| Diet.f mixed diet                 | -0.0019707 | 0.0049861 | -0.395 | 0.693 |
| Diet.f phyto diet                 | 0.0024402  | 0.0048988 | 0.498  | 0.619 |
| Stress.f stress                   | 0.0003853  | 0.0045176 | 0.085  | 0.932 |
| Age.f 4 months:sexj               | -0.0008106 | 0.0054524 | -0.149 | 0.882 |
| Age.f 7 months:sexj               | NA         | NA        | NA     | NA    |
| Age.f 4 months:sexm               | 0.0004840  | 0.0037945 | 0.128  | 0.899 |
| Age.f 7 months:sexm               | -0.0005626 | 0.0042692 | -0.132 | 0.895 |
| Age.f 4 months:Temp.fWarm         | -0.0018787 | 0.0034470 | -0.545 | 0.586 |
| Age.f 7 months:Temp.fWarm         | -0.0034041 | 0.0049063 | -0.694 | 0.488 |
| Age.f 4 months:Diet.f mixed diet  | 0.0002952  | 0.0044932 | 0.066  | 0.948 |
| Age.f 7 months:Diet.f mixed diet  | -0.0024498 | 0.0069396 | -0.353 | 0.724 |
| Age.f 4 months:Diet.f phyto diet  | 0.0023150  | 0.0043104 | 0.537  | 0.592 |
| Age.f 7 months:Diet.f phyto diet  | 0.0013335  | 0.0071390 | 0.187  | 0.852 |
| Age.f 4 months:Stress.f stress    | -0.0035418 | 0.0033896 | -1.045 | 0.297 |
| Age.f 7 months:Stress.f stress    | -0.0032960 | 0.0048449 | -0.680 | 0.497 |
| sexj:Temp.fWarm                   | 0.0038815  | 0.0045892 | 0.846  | 0.399 |
| sexm:Temp.fWarm                   | 0.0012978  | 0.0032672 | 0.397  | 0.692 |
| sexj:Diet.f mixed diet            | -0.0041726 | 0.0057020 | -0.732 | 0.465 |
| sexm:Diet.f mixed diet            | 0.0016449  | 0.0044521 | 0.369  | 0.712 |
| sexj:Diet.f phyto diet            | -0.0054634 | 0.0054868 | -0.996 | 0.320 |
| sexm:Diet.f phyto diet            | -0.0009177 | 0.0044097 | -0.208 | 0.835 |
| sexj:Stress.f stress              | 0.0023015  | 0.0044063 | 0.522  | 0.602 |
| sexm:Stress.f stress              | 0.0007384  | 0.0032739 | 0.226  | 0.822 |
| Temp.fWarm:Diet.f mixed diet      | 0.0004029  | 0.0039178 | 0.103  | 0.918 |
| Temp.fWarm:Diet.f phyto diet      | -0.0051528 | 0.0037939 | -1.358 | 0.176 |
| Temp.fWarm:Stress.f stress        | 0.0046136  | 0.0030531 | 1.511  | 0.132 |
| Diet.f mixed diet:Stress.f stress | 0.0018420  | 0.0038211 | 0.482  | 0.630 |
| Diet.f phyto diet:Stress.f stress | -0.0003640 | 0.0038289 | -0.095 | 0.924 |

Residual standard error: 0.01109 on 229 degrees of freedom  
Multiple R-squared: 0.09709, Adjusted R-squared: -0.02908  
F-statistic: 0.7696 on 32 and 229 DF, p-value: 0.8104

```
>
> # reduced model
>
> lm.03 <- lm(formula=wetwteyes ~ Age.f+Temp.f+
+ Diet.f+Age.f:Temp.f+Age.f:Diet.f+Temp.f:Diet.f, data=data.df)
>
> anova(lm.03)
Analysis of Variance Table
```

```
Response: wetwteyes
      Df Sum Sq Mean Sq F value Pr(>F)
Age.f    2 0.0001626 0.0000813 0.6809 0.5071
```

```
Temp.f      1 0.0000779 0.0000779 0.6522 0.4201
Diet.f      2 0.0001929 0.0000964 0.8076 0.4471
Age.f:Temp.f 2 0.0002829 0.0001414 1.1846 0.3076
Age.f:Diet.f 4 0.0003395 0.0000849 0.7109 0.5851
Temp.f:Diet.f 2 0.0005421 0.0002711 2.2704 0.1054
Residuals   248 0.0296093 0.0001194
> summary(lm.03)
```

Call:

```
lm(formula = wetwteyes ~ Age.f + Temp.f + Diet.f + Age.f:Temp.f +
    Age.f:Diet.f + Temp.f:Diet.f, data = data.df)
```

Residuals:

```
      Min       1Q   Median       3Q      Max
-0.0085549 -0.0023962 -0.0011025  0.0004346  0.1308451
```

Coefficients:

|                                  | Estimate   | Std. Error | t value | Pr(> t )   |
|----------------------------------|------------|------------|---------|------------|
| (Intercept)                      | 0.0068601  | 0.0022674  | 3.026   | 0.00274 ** |
| Age.f 4 months                   | -0.0019729 | 0.0033717  | -0.585  | 0.55899    |
| Age.f 7 months                   | -0.0013969 | 0.0060400  | -0.231  | 0.81730    |
| Temp.fWarm                       | 0.0052948  | 0.0029368  | 1.803   | 0.07262 .  |
| Diet.f mixed diet                | -0.0021816 | 0.0030472  | -0.716  | 0.47471    |
| Diet.f phyto diet                | -0.0002837 | 0.0030257  | -0.094  | 0.92538    |
| Age.f 4 months:Temp.fWarm        | -0.0032792 | 0.0031075  | -1.055  | 0.29233    |
| Age.f 7 months:Temp.fWarm        | -0.0045213 | 0.0038201  | -1.184  | 0.23772    |
| Age.f 4 months:Diet.f mixed diet | 0.0011598  | 0.0040229  | 0.288   | 0.77336    |
| Age.f 7 months:Diet.f mixed diet | 0.0015045  | 0.0063341  | 0.238   | 0.81245    |
| Age.f 4 months:Diet.f phyto diet | 0.0043975  | 0.0038632  | 1.138   | 0.25609    |
| Age.f 7 months:Diet.f phyto diet | 0.0064256  | 0.0065186  | 0.986   | 0.32522    |
| Temp.fWarm:Diet.f mixed diet     | -0.0001418 | 0.0036418  | -0.039  | 0.96896    |
| Temp.fWarm:Diet.f phyto diet     | -0.0061227 | 0.0035694  | -1.715  | 0.08753 .  |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 0.01093 on 248 degrees of freedom

Multiple R-squared: 0.0512, Adjusted R-squared: 0.001465

F-statistic: 1.029 on 13 and 248 DF, p-value: 0.4234

>

>

> # Rest of code requires asreml license:

>

> library(asreml)

Loading required package: grid

>

```
> asreml.mv <- asreml(fixed = cbind(pigment.3, wetwteyes) ~ trait +
    trait:Age.f + trait:Temp.f +
    + trait:Age.f:Temp.f,
    + rcov = ~ units:us(trait), data = data.df, na.method.Y = "exclude",
```

```
+ na.method.X = "exclude", maxiter=30)
```

asreml(): 2.00 Library: 2.00bb Run: Thu Oct 09 09:41:59 2008

Licensed to: Australian Antarctic Division

Serial Number: 402061977 Expires: 30-sep-2009 (356 days)

Equations: 24 (24 dense)

Initial update shrinkage factor: 0.316

12 singularities detected in design matrix

| LogLik    | S2     | DF  |          |
|-----------|--------|-----|----------|
| 623.0277  | 1.0000 | 512 | 09:41:59 |
| 961.8813  | 1.0000 | 512 | 09:41:59 |
| 1277.3204 | 1.0000 | 512 | 09:41:59 |
| 1533.8540 | 1.0000 | 512 | 09:41:59 |
| 1630.9999 | 1.0000 | 512 | 09:41:59 |
| 1658.2771 | 1.0000 | 512 | 09:41:59 |
| 1662.3060 | 1.0000 | 512 | 09:41:59 |
| 1662.4582 | 1.0000 | 512 | 09:41:59 |
| 1662.4585 | 1.0000 | 512 | 09:41:59 |

Finished on: Thu Oct 09 09:41:59 2008

LogLikelihood Converged

>

>

> anova(asreml.mv)

Wald tests for fixed effects

Response: cbind(pigment.3, wetwteyes)

Terms added sequentially; adjusted for those above

|                    | Df | Sum of Sq | Wald statistic | Pr(Chisq)     |
|--------------------|----|-----------|----------------|---------------|
| trait              | 2  | 1806.50   | 1806.50        | < 2.2e-16 *** |
| trait:Age.f        | 4  | 39.70     | 39.70          | 5.004e-08 *** |
| trait:Temp.f       | 2  | 4.52      | 4.52           | 0.1042        |
| trait:Age.f:Temp.f | 4  | 38.46     | 38.46          | 8.988e-08 *** |
| residual (MS)      |    | 1.00      |                |               |

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

> (summary(asreml.mv))\$varcomp

|                             | gamma        | component    | std.error    | z.ratio   |
|-----------------------------|--------------|--------------|--------------|-----------|
| R!variance                  | 1.000000e+00 | 1.000000e+00 | NA           | NA        |
| R!trait.pigment.3:pigment.3 | 2.181000e-03 | 2.181000e-03 | 1.927735e-04 | 11.313791 |
| R!trait.wetwteyes:pigment.3 | 3.831791e-05 | 3.831791e-05 | 3.206075e-05 | 1.195166  |
| R!trait.wetwteyes:wetwteyes | 1.199789e-04 | 1.199789e-04 | 1.060473e-05 | 11.313709 |

constraint

```

R!variance                Fixed
R!trait.pigment.3:pigment.3 Unconstrained
R!trait.wetwteyes:pigment.3 Unconstrained
R!trait.wetwteyes:wetwteyes Unconstrained
> (summary(asreml.mv))$coef.fixed
              solution  std error
trait_pigment.3:Age.f_1 month:Temp.f_Cold  0.000000e+00    NA
trait_pigment.3:Age.f_1 month:Temp.f_Warm  0.000000e+00    NA
trait_pigment.3:Age.f_4 months:Temp.f_Cold  0.000000e+00    NA
trait_pigment.3:Age.f_4 months:Temp.f_Warm  3.799366e-04 0.013070736
trait_pigment.3:Age.f_7 months:Temp.f_Cold  0.000000e+00    NA
trait_pigment.3:Age.f_7 months:Temp.f_Warm -8.967892e-02 0.015808383
trait_wetwteyes:Age.f_1 month:Temp.f_Cold  0.000000e+00    NA
trait_wetwteyes:Age.f_1 month:Temp.f_Warm  0.000000e+00    NA
trait_wetwteyes:Age.f_4 months:Temp.f_Cold  0.000000e+00    NA
trait_wetwteyes:Age.f_4 months:Temp.f_Warm -3.723167e-03 0.003065672
trait_wetwteyes:Age.f_7 months:Temp.f_Cold  0.000000e+00    NA
trait_wetwteyes:Age.f_7 months:Temp.f_Warm -4.331042e-03 0.003707773
trait_pigment.3:Temp.f_Cold                0.000000e+00    NA
trait_pigment.3:Temp.f_Warm                5.915174e-03 0.008526408
trait_wetwteyes:Temp.f_Cold                0.000000e+00    NA
trait_wetwteyes:Temp.f_Warm                3.206667e-03 0.001999824
trait_pigment.3:Age.f_1 month              0.000000e+00    NA
trait_pigment.3:Age.f_4 months             -2.004605e-02 0.008942572
trait_pigment.3:Age.f_7 months             6.536813e-02 0.010222804
trait_wetwteyes:Age.f_1 month              0.000000e+00    NA
trait_wetwteyes:Age.f_4 months             8.566667e-05 0.002097433
trait_wetwteyes:Age.f_7 months             9.210417e-04 0.002397705
trait_pigment.3                          1.189660e-01 0.006029081
trait_wetwteyes                          6.038333e-03 0.001414089
              z ratio
trait_pigment.3:Age.f_1 month:Temp.f_Cold    NA
trait_pigment.3:Age.f_1 month:Temp.f_Warm    NA
trait_pigment.3:Age.f_4 months:Temp.f_Cold    NA
trait_pigment.3:Age.f_4 months:Temp.f_Warm  0.02906772
trait_pigment.3:Age.f_7 months:Temp.f_Cold    NA
trait_pigment.3:Age.f_7 months:Temp.f_Warm -5.67287107
trait_wetwteyes:Age.f_1 month:Temp.f_Cold    NA
trait_wetwteyes:Age.f_1 month:Temp.f_Warm    NA
trait_wetwteyes:Age.f_4 months:Temp.f_Cold    NA
trait_wetwteyes:Age.f_4 months:Temp.f_Warm -1.21446999
trait_wetwteyes:Age.f_7 months:Temp.f_Cold    NA
trait_wetwteyes:Age.f_7 months:Temp.f_Warm -1.16809792
trait_pigment.3:Temp.f_Cold                  NA
trait_pigment.3:Temp.f_Warm                  0.69374750
trait_wetwteyes:Temp.f_Cold                  NA
trait_wetwteyes:Temp.f_Warm                  1.60347451
trait_pigment.3:Age.f_1 month                NA
trait_pigment.3:Age.f_4 months              -2.24164218
trait_pigment.3:Age.f_7 months               6.39434452

```

```

trait_wetwteyes:Age.f_ 1 month          NA
trait_wetwteyes:Age.f_ 4 months        0.04084358
trait_wetwteyes:Age.f_ 7 months        0.38413475
trait_pigment.3                        19.73203746
trait_wetwteyes                        4.27012240
>
> vc <- ((summary(asreml.mv))$varcomp)[[1]]
>
> vc[3]/((vc[2]*vc[4])^0.5)
[1] 0.07490686
> asreml.mv <- asreml(fixed = cbind(pigment.3, wetwteyes) ~ trait +
+   trait:Age.f + trait:Temp.f +
+   trait:Age.f:Temp.f + trait:Diet.f + trait:Diet.f:Temp.f + trait:Age.f:Diet.f:Temp.f,
+   rcov = ~ units:us(trait), data = data.df, na.method.Y = "exclude",
+   na.method.X = "exclude", maxiter = 30)

```

asreml(): 2.00 Library: 2.00bb Run: Thu Oct 09 09:42:08 2008  
 Licensed to: Australian Antarctic Division  
 Serial Number: 402061977 Expires: 30-sep-2009 (356 days)

Equations: 78 (78 dense)  
 Initial update shrinkage factor: 0.316  
 42 singularities detected in design matrix

| LogLik    | S2     | DF  |          |
|-----------|--------|-----|----------|
| 770.6042  | 1.0000 | 488 | 09:42:08 |
| 1047.1874 | 1.0000 | 488 | 09:42:08 |
| 1293.6619 | 1.0000 | 488 | 09:42:08 |
| 1491.0057 | 1.0000 | 488 | 09:42:08 |
| 1561.9320 | 1.0000 | 488 | 09:42:08 |
| 1579.7414 | 1.0000 | 488 | 09:42:08 |
| 1581.8123 | 1.0000 | 488 | 09:42:08 |
| 1581.8595 | 1.0000 | 488 | 09:42:08 |
| 1581.8596 | 1.0000 | 488 | 09:42:08 |

Finished on: Thu Oct 09 09:42:08 2008

LogLikelihood Converged

>

> anova(asreml.mv)

Wald tests for fixed effects

Response: cbind(pigment.3, wetwteyes)

Terms added sequentially; adjusted for those above

|             | Df | Sum of Sq | Wald statistic | Pr(Chisq)     |
|-------------|----|-----------|----------------|---------------|
| trait       | 2  | 2187.65   | 2187.65        | < 2.2e-16 *** |
| trait:Age.f | 4  | 47.35     | 47.35          | 1.288e-09 *** |

```

trait:Temp.f      2    5.06      5.06 0.07964 .
trait:Diet.f      4    6.47      6.47 0.16651
trait:Age.f:Temp.f  4   46.96     46.96 1.555e-09 ***
trait:Temp.f:Diet.f  4   40.69     40.69 3.119e-08 ***
trait:Age.f:Temp.f:Diet.f 16  25.20     25.20 0.06644 .
residual (MS)                1.00

```

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

```
> (summary(asreml.mv))$varcomp
```

```

              gamma component std.error z.ratio
R!variance      1.000000e+00 1.000000e+00      NA      NA
R!trait.pigment.3:pigment.3 1.820481e-03 1.820481e-03 1.648184e-04 11.045370
R!trait.wetwteyes:pigment.3 1.064466e-05 1.064466e-05 2.997154e-05 0.355159
R!trait.wetwteyes:wetwteyes 1.203366e-04 1.203366e-04 1.089477e-05
11.045361

```

constraint

```

R!variance      Fixed
R!trait.pigment.3:pigment.3 Unconstrained
R!trait.wetwteyes:pigment.3 Unconstrained
R!trait.wetwteyes:wetwteyes Unconstrained
> (summary(asreml.mv))$coef.fixed

```

solution

```

trait_pigment.3:Age.f_1 month:Temp.f_Cold:Diet.f_limited diet 0.000000000
trait_pigment.3:Age.f_1 month:Temp.f_Cold:Diet.f_mixed diet 0.000000000
trait_pigment.3:Age.f_1 month:Temp.f_Cold:Diet.f_phyto diet 0.000000000
trait_pigment.3:Age.f_1 month:Temp.f_Warm:Diet.f_limited diet 0.000000000
trait_pigment.3:Age.f_1 month:Temp.f_Warm:Diet.f_mixed diet 0.000000000
trait_pigment.3:Age.f_1 month:Temp.f_Warm:Diet.f_phyto diet 0.000000000
trait_pigment.3:Age.f_4 months:Temp.f_Cold:Diet.f_limited diet 0.000000000
trait_pigment.3:Age.f_4 months:Temp.f_Cold:Diet.f_mixed diet -0.038488279
trait_pigment.3:Age.f_4 months:Temp.f_Cold:Diet.f_phyto diet -0.043219774
trait_pigment.3:Age.f_4 months:Temp.f_Warm:Diet.f_limited diet 0.000000000
trait_pigment.3:Age.f_4 months:Temp.f_Warm:Diet.f_mixed diet -0.022557289
trait_pigment.3:Age.f_4 months:Temp.f_Warm:Diet.f_phyto diet -0.013573040
trait_pigment.3:Age.f_7 months:Temp.f_Cold:Diet.f_limited diet 0.000000000
trait_pigment.3:Age.f_7 months:Temp.f_Cold:Diet.f_mixed diet 0.094723542
trait_pigment.3:Age.f_7 months:Temp.f_Cold:Diet.f_phyto diet 0.114060998
trait_pigment.3:Age.f_7 months:Temp.f_Warm:Diet.f_limited diet 0.000000000
trait_pigment.3:Age.f_7 months:Temp.f_Warm:Diet.f_mixed diet -0.035729002
trait_pigment.3:Age.f_7 months:Temp.f_Warm:Diet.f_phyto diet -0.016048019
trait_wetwteyes:Age.f_1 month:Temp.f_Cold:Diet.f_limited diet 0.000000000
trait_wetwteyes:Age.f_1 month:Temp.f_Cold:Diet.f_mixed diet 0.000000000
trait_wetwteyes:Age.f_1 month:Temp.f_Cold:Diet.f_phyto diet 0.000000000
trait_wetwteyes:Age.f_1 month:Temp.f_Warm:Diet.f_limited diet 0.000000000
trait_wetwteyes:Age.f_1 month:Temp.f_Warm:Diet.f_mixed diet 0.000000000
trait_wetwteyes:Age.f_1 month:Temp.f_Warm:Diet.f_phyto diet 0.000000000
trait_wetwteyes:Age.f_4 months:Temp.f_Cold:Diet.f_limited diet 0.000000000
trait_wetwteyes:Age.f_4 months:Temp.f_Cold:Diet.f_mixed diet -0.001170000
trait_wetwteyes:Age.f_4 months:Temp.f_Cold:Diet.f_phyto diet -0.000940000
trait_wetwteyes:Age.f_4 months:Temp.f_Warm:Diet.f_limited diet 0.000000000

```



|                                                                  |              |
|------------------------------------------------------------------|--------------|
| trait_wetwteyes:Age.f_ 4 months:Temp.f_Warm:Diet.f_ mixed diet   |              |
| 0.003315000                                                      |              |
| trait_wetwteyes:Age.f_ 4 months:Temp.f_Warm:Diet.f_ phyto diet   | 0.009735000  |
| trait_wetwteyes:Age.f_ 7 months:Temp.f_Cold:Diet.f_ limited diet | 0.000000000  |
| trait_wetwteyes:Age.f_ 7 months:Temp.f_Cold:Diet.f_ mixed diet   | 0.002215000  |
| trait_wetwteyes:Age.f_ 7 months:Temp.f_Cold:Diet.f_ phyto diet   | 0.005050000  |
| trait_wetwteyes:Age.f_ 7 months:Temp.f_Warm:Diet.f_ limited diet | 0.000000000  |
| trait_wetwteyes:Age.f_ 7 months:Temp.f_Warm:Diet.f_ mixed diet   | 0.000585000  |
| trait_wetwteyes:Age.f_ 7 months:Temp.f_Warm:Diet.f_ phyto diet   | 0.007855000  |
| trait_pigment.3:Temp.f_Cold:Diet.f_ limited diet                 | 0.000000000  |
| trait_pigment.3:Temp.f_Cold:Diet.f_ mixed diet                   | 0.000000000  |
| trait_pigment.3:Temp.f_Cold:Diet.f_ phyto diet                   | 0.000000000  |
| trait_pigment.3:Temp.f_Warm:Diet.f_ limited diet                 | 0.000000000  |
| trait_pigment.3:Temp.f_Warm:Diet.f_ mixed diet                   | -0.039310780 |
| trait_pigment.3:Temp.f_Warm:Diet.f_ phyto diet                   | -0.083653729 |
| trait_wetwteyes:Temp.f_Cold:Diet.f_ limited diet                 | 0.000000000  |
| trait_wetwteyes:Temp.f_Cold:Diet.f_ mixed diet                   | 0.000000000  |
| trait_wetwteyes:Temp.f_Cold:Diet.f_ phyto diet                   | 0.000000000  |
| trait_wetwteyes:Temp.f_Warm:Diet.f_ limited diet                 | 0.000000000  |
| trait_wetwteyes:Temp.f_Warm:Diet.f_ mixed diet                   | -0.001165000 |
| trait_wetwteyes:Temp.f_Warm:Diet.f_ phyto diet                   | -0.010470000 |
| trait_pigment.3:Age.f_ 1 month:Temp.f_Cold                       | 0.000000000  |
| trait_pigment.3:Age.f_ 1 month:Temp.f_Warm                       | 0.000000000  |
| trait_pigment.3:Age.f_ 4 months:Temp.f_Cold                      | 0.000000000  |
| trait_pigment.3:Age.f_ 4 months:Temp.f_Warm                      | -0.011045508 |
| trait_pigment.3:Age.f_ 7 months:Temp.f_Cold                      | 0.000000000  |
| trait_pigment.3:Age.f_ 7 months:Temp.f_Warm                      | 0.039816383  |
| trait_wetwteyes:Age.f_ 1 month:Temp.f_Cold                       | 0.000000000  |
| trait_wetwteyes:Age.f_ 1 month:Temp.f_Warm                       | 0.000000000  |
| trait_wetwteyes:Age.f_ 4 months:Temp.f_Cold                      | 0.000000000  |
| trait_wetwteyes:Age.f_ 4 months:Temp.f_Warm                      | -0.009055000 |
| trait_wetwteyes:Age.f_ 7 months:Temp.f_Cold                      | 0.000000000  |
| trait_wetwteyes:Age.f_ 7 months:Temp.f_Warm                      | -0.004285000 |
| trait_pigment.3:Diet.f_ limited diet                             | 0.000000000  |
| trait_pigment.3:Diet.f_ mixed diet                               | 0.025318953  |
| trait_pigment.3:Diet.f_ phyto diet                               | 0.056322989  |
| trait_wetwteyes:Diet.f_ limited diet                             | 0.000000000  |
| trait_wetwteyes:Diet.f_ mixed diet                               | -0.001670000 |
| trait_wetwteyes:Diet.f_ phyto diet                               | 0.001890000  |
| trait_pigment.3:Temp.f_Cold                                      | 0.000000000  |
| trait_pigment.3:Temp.f_Warm                                      | 0.046903344  |
| trait_wetwteyes:Temp.f_Cold                                      | 0.000000000  |
| trait_wetwteyes:Temp.f_Warm                                      | 0.007085000  |
| trait_pigment.3:Age.f_ 1 month                                   | 0.000000000  |
| trait_pigment.3:Age.f_ 4 months                                  | 0.007194379  |
| trait_pigment.3:Age.f_ 7 months                                  | -0.035689444 |
| trait_wetwteyes:Age.f_ 1 month                                   | 0.000000000  |
| trait_wetwteyes:Age.f_ 4 months                                  | 0.000915000  |
| trait_wetwteyes:Age.f_ 7 months                                  | -0.001515000 |

|                                                                |             |
|----------------------------------------------------------------|-------------|
| trait_pigment.3                                                | 0.091752069 |
| trait_wetwteyes                                                | 0.005965000 |
| std error                                                      |             |
| trait_pigment.3:Age.f_1 month:Temp.f_Cold:Diet.f_limited diet  | NA          |
| trait_pigment.3:Age.f_1 month:Temp.f_Cold:Diet.f_mixed diet    | NA          |
| trait_pigment.3:Age.f_1 month:Temp.f_Cold:Diet.f_phyto diet    | NA          |
| trait_pigment.3:Age.f_1 month:Temp.f_Warm:Diet.f_limited diet  | NA          |
| trait_pigment.3:Age.f_1 month:Temp.f_Warm:Diet.f_mixed diet    | NA          |
| trait_pigment.3:Age.f_1 month:Temp.f_Warm:Diet.f_phyto diet    | NA          |
| trait_pigment.3:Age.f_4 months:Temp.f_Cold:Diet.f_limited diet | NA          |
| trait_pigment.3:Age.f_4 months:Temp.f_Cold:Diet.f_mixed diet   | 0.021333539 |
| trait_pigment.3:Age.f_4 months:Temp.f_Cold:Diet.f_phyto diet   | 0.021333539 |
| trait_pigment.3:Age.f_4 months:Temp.f_Warm:Diet.f_limited diet | NA          |
| trait_pigment.3:Age.f_4 months:Temp.f_Warm:Diet.f_mixed diet   | 0.023369721 |
| trait_pigment.3:Age.f_4 months:Temp.f_Warm:Diet.f_phyto diet   | 0.021333539 |
| trait_pigment.3:Age.f_7 months:Temp.f_Cold:Diet.f_limited diet | NA          |
| trait_pigment.3:Age.f_7 months:Temp.f_Cold:Diet.f_mixed diet   | 0.034399298 |
| trait_pigment.3:Age.f_7 months:Temp.f_Cold:Diet.f_phyto diet   | 0.035697839 |
| trait_pigment.3:Age.f_7 months:Temp.f_Warm:Diet.f_limited diet | NA          |
| trait_pigment.3:Age.f_7 months:Temp.f_Warm:Diet.f_mixed diet   | 0.035697839 |
| trait_pigment.3:Age.f_7 months:Temp.f_Warm:Diet.f_phyto diet   | 0.036329708 |
| trait_wetwteyes:Age.f_1 month:Temp.f_Cold:Diet.f_limited diet  | NA          |
| trait_wetwteyes:Age.f_1 month:Temp.f_Cold:Diet.f_mixed diet    | NA          |
| trait_wetwteyes:Age.f_1 month:Temp.f_Cold:Diet.f_phyto diet    | NA          |
| trait_wetwteyes:Age.f_1 month:Temp.f_Warm:Diet.f_limited diet  | NA          |
| trait_wetwteyes:Age.f_1 month:Temp.f_Warm:Diet.f_mixed diet    | NA          |
| trait_wetwteyes:Age.f_1 month:Temp.f_Warm:Diet.f_phyto diet    | NA          |
| trait_wetwteyes:Age.f_4 months:Temp.f_Cold:Diet.f_limited diet | NA          |
| trait_wetwteyes:Age.f_4 months:Temp.f_Cold:Diet.f_mixed diet   | 0.005484902 |
| trait_wetwteyes:Age.f_4 months:Temp.f_Cold:Diet.f_phyto diet   | 0.005484902 |
| trait_wetwteyes:Age.f_4 months:Temp.f_Warm:Diet.f_limited diet | NA          |
| trait_wetwteyes:Age.f_4 months:Temp.f_Warm:Diet.f_mixed diet   | 0.006008410 |
| trait_wetwteyes:Age.f_4 months:Temp.f_Warm:Diet.f_phyto diet   | 0.005484902 |
| trait_wetwteyes:Age.f_7 months:Temp.f_Cold:Diet.f_limited diet | NA          |
| trait_wetwteyes:Age.f_7 months:Temp.f_Cold:Diet.f_mixed diet   | 0.008844139 |
| trait_wetwteyes:Age.f_7 months:Temp.f_Cold:Diet.f_phyto diet   | 0.009177997 |
| trait_wetwteyes:Age.f_7 months:Temp.f_Warm:Diet.f_limited diet | NA          |
| trait_wetwteyes:Age.f_7 months:Temp.f_Warm:Diet.f_mixed diet   | 0.009177997 |
| trait_wetwteyes:Age.f_7 months:Temp.f_Warm:Diet.f_phyto diet   | 0.009340452 |
| trait_pigment.3:Temp.f_Cold:Diet.f_limited diet                | NA          |
| trait_pigment.3:Temp.f_Cold:Diet.f_mixed diet                  | NA          |
| trait_pigment.3:Temp.f_Cold:Diet.f_phyto diet                  | NA          |
| trait_pigment.3:Temp.f_Warm:Diet.f_limited diet                | NA          |
| trait_pigment.3:Temp.f_Warm:Diet.f_mixed diet                  | 0.019081297 |
| trait_pigment.3:Temp.f_Warm:Diet.f_phyto diet                  | 0.019081297 |
| trait_wetwteyes:Temp.f_Cold:Diet.f_limited diet                | NA          |
| trait_wetwteyes:Temp.f_Cold:Diet.f_mixed diet                  | NA          |
| trait_wetwteyes:Temp.f_Cold:Diet.f_phyto diet                  | NA          |
| trait_wetwteyes:Temp.f_Warm:Diet.f_limited diet                | NA          |
| trait_wetwteyes:Temp.f_Warm:Diet.f_mixed diet                  | 0.004905846 |

|                                               |             |
|-----------------------------------------------|-------------|
| trait_wetwteyes:Temp.f_Warm:Diet.f_phyto diet | 0.004905846 |
| trait_pigment.3:Age.f_1 month:Temp.f_Cold     | NA          |
| trait_pigment.3:Age.f_1 month:Temp.f_Warm     | NA          |
| trait_pigment.3:Age.f_4 months:Temp.f_Cold    | NA          |
| trait_pigment.3:Age.f_4 months:Temp.f_Warm    | 0.023369721 |
| trait_pigment.3:Age.f_7 months:Temp.f_Cold    | NA          |
| trait_pigment.3:Age.f_7 months:Temp.f_Warm    | 0.044749609 |
| trait_wetwteyes:Age.f_1 month:Temp.f_Cold     | NA          |
| trait_wetwteyes:Age.f_1 month:Temp.f_Warm     | NA          |
| trait_wetwteyes:Age.f_4 months:Temp.f_Cold    | NA          |
| trait_wetwteyes:Age.f_4 months:Temp.f_Warm    | 0.006008410 |
| trait_wetwteyes:Age.f_7 months:Temp.f_Cold    | NA          |
| trait_wetwteyes:Age.f_7 months:Temp.f_Warm    | 0.011505228 |
| trait_pigment.3:Diet.f_limited diet           | NA          |
| trait_pigment.3:Diet.f_mixed diet             | 0.013492515 |
| trait_pigment.3:Diet.f_phyto diet             | 0.013492515 |
| trait_wetwteyes:Diet.f_limited diet           | NA          |
| trait_wetwteyes:Diet.f_mixed diet             | 0.003468957 |
| trait_wetwteyes:Diet.f_phyto diet             | 0.003468957 |
| trait_pigment.3:Temp.f_Cold                   | NA          |
| trait_pigment.3:Temp.f_Warm                   | 0.013492515 |
| trait_wetwteyes:Temp.f_Cold                   | NA          |
| trait_wetwteyes:Temp.f_Warm                   | 0.003468957 |
| trait_pigment.3:Age.f_1 month                 | NA          |
| trait_pigment.3:Age.f_4 months                | 0.016524888 |
| trait_pigment.3:Age.f_7 months                | 0.031642752 |
| trait_wetwteyes:Age.f_1 month                 | NA          |
| trait_wetwteyes:Age.f_4 months                | 0.004248587 |
| trait_wetwteyes:Age.f_7 months                | 0.008135425 |
| trait_pigment.3                               | 0.009540649 |
| trait_wetwteyes                               | 0.002452923 |

z ratio

|                                                                |            |
|----------------------------------------------------------------|------------|
| trait_pigment.3:Age.f_1 month:Temp.f_Cold:Diet.f_limited diet  | NA         |
| trait_pigment.3:Age.f_1 month:Temp.f_Cold:Diet.f_mixed diet    | NA         |
| trait_pigment.3:Age.f_1 month:Temp.f_Cold:Diet.f_phyto diet    | NA         |
| trait_pigment.3:Age.f_1 month:Temp.f_Warm:Diet.f_limited diet  | NA         |
| trait_pigment.3:Age.f_1 month:Temp.f_Warm:Diet.f_mixed diet    | NA         |
| trait_pigment.3:Age.f_1 month:Temp.f_Warm:Diet.f_phyto diet    | NA         |
| trait_pigment.3:Age.f_4 months:Temp.f_Cold:Diet.f_limited diet | NA         |
| trait_pigment.3:Age.f_4 months:Temp.f_Cold:Diet.f_mixed diet   | -1.8041207 |
| trait_pigment.3:Age.f_4 months:Temp.f_Cold:Diet.f_phyto diet   | -2.0259074 |
| trait_pigment.3:Age.f_4 months:Temp.f_Warm:Diet.f_limited diet | NA         |
| trait_pigment.3:Age.f_4 months:Temp.f_Warm:Diet.f_mixed diet   | -0.9652357 |
| trait_pigment.3:Age.f_4 months:Temp.f_Warm:Diet.f_phyto diet   | -0.6362301 |
| trait_pigment.3:Age.f_7 months:Temp.f_Cold:Diet.f_limited diet | NA         |
| trait_pigment.3:Age.f_7 months:Temp.f_Cold:Diet.f_mixed diet   | 2.7536475  |
| trait_pigment.3:Age.f_7 months:Temp.f_Cold:Diet.f_phyto diet   | 3.1951794  |
| trait_pigment.3:Age.f_7 months:Temp.f_Warm:Diet.f_limited diet | NA         |
| trait_pigment.3:Age.f_7 months:Temp.f_Warm:Diet.f_mixed diet   | -1.0008730 |
| trait_pigment.3:Age.f_7 months:Temp.f_Warm:Diet.f_phyto diet   | -0.4417327 |

|                                                                |            |
|----------------------------------------------------------------|------------|
| trait_wetwteyes:Age.f_1 month:Temp.f_Cold:Diet.f_limited diet  | NA         |
| trait_wetwteyes:Age.f_1 month:Temp.f_Cold:Diet.f_mixed diet    | NA         |
| trait_wetwteyes:Age.f_1 month:Temp.f_Cold:Diet.f_phyto diet    | NA         |
| trait_wetwteyes:Age.f_1 month:Temp.f_Warm:Diet.f_limited diet  | NA         |
| trait_wetwteyes:Age.f_1 month:Temp.f_Warm:Diet.f_mixed diet    | NA         |
| trait_wetwteyes:Age.f_1 month:Temp.f_Warm:Diet.f_phyto diet    | NA         |
| trait_wetwteyes:Age.f_4 months:Temp.f_Cold:Diet.f_limited diet | NA         |
| trait_wetwteyes:Age.f_4 months:Temp.f_Cold:Diet.f_mixed diet   | -0.2133128 |
| trait_wetwteyes:Age.f_4 months:Temp.f_Cold:Diet.f_phyto diet   | -0.1713795 |
| trait_wetwteyes:Age.f_4 months:Temp.f_Warm:Diet.f_limited diet | NA         |
| trait_wetwteyes:Age.f_4 months:Temp.f_Warm:Diet.f_mixed diet   | 0.5517267  |
| trait_wetwteyes:Age.f_4 months:Temp.f_Warm:Diet.f_phyto diet   | 1.7748721  |
| trait_wetwteyes:Age.f_7 months:Temp.f_Cold:Diet.f_limited diet | NA         |
| trait_wetwteyes:Age.f_7 months:Temp.f_Cold:Diet.f_mixed diet   | 0.2504483  |
| trait_wetwteyes:Age.f_7 months:Temp.f_Cold:Diet.f_phyto diet   | 0.5502290  |
| trait_wetwteyes:Age.f_7 months:Temp.f_Warm:Diet.f_limited diet | NA         |
| trait_wetwteyes:Age.f_7 months:Temp.f_Warm:Diet.f_mixed diet   | 0.0637394  |
| trait_wetwteyes:Age.f_7 months:Temp.f_Warm:Diet.f_phyto diet   | 0.8409657  |
| trait_pigment.3:Temp.f_Cold:Diet.f_limited diet                | NA         |
| trait_pigment.3:Temp.f_Cold:Diet.f_mixed diet                  | NA         |
| trait_pigment.3:Temp.f_Cold:Diet.f_phyto diet                  | NA         |
| trait_pigment.3:Temp.f_Warm:Diet.f_limited diet                | NA         |
| trait_pigment.3:Temp.f_Warm:Diet.f_mixed diet                  | -2.0601733 |
| trait_pigment.3:Temp.f_Warm:Diet.f_phyto diet                  | -4.3840692 |
| trait_wetwteyes:Temp.f_Cold:Diet.f_limited diet                | NA         |
| trait_wetwteyes:Temp.f_Cold:Diet.f_mixed diet                  | NA         |
| trait_wetwteyes:Temp.f_Cold:Diet.f_phyto diet                  | NA         |
| trait_wetwteyes:Temp.f_Warm:Diet.f_limited diet                | NA         |
| trait_wetwteyes:Temp.f_Warm:Diet.f_mixed diet                  | -0.2374718 |
| trait_wetwteyes:Temp.f_Warm:Diet.f_phyto diet                  | -2.1341885 |
| trait_pigment.3:Age.f_1 month:Temp.f_Cold                      | NA         |
| trait_pigment.3:Age.f_1 month:Temp.f_Warm                      | NA         |
| trait_pigment.3:Age.f_4 months:Temp.f_Cold                     | NA         |
| trait_pigment.3:Age.f_4 months:Temp.f_Warm                     | -0.4726419 |
| trait_pigment.3:Age.f_7 months:Temp.f_Cold                     | NA         |
| trait_pigment.3:Age.f_7 months:Temp.f_Warm                     | 0.8897593  |
| trait_wetwteyes:Age.f_1 month:Temp.f_Cold                      | NA         |
| trait_wetwteyes:Age.f_1 month:Temp.f_Warm                      | NA         |
| trait_wetwteyes:Age.f_4 months:Temp.f_Cold                     | NA         |
| trait_wetwteyes:Age.f_4 months:Temp.f_Warm                     | -1.5070544 |
| trait_wetwteyes:Age.f_7 months:Temp.f_Cold                     | NA         |
| trait_wetwteyes:Age.f_7 months:Temp.f_Warm                     | -0.3724394 |
| trait_pigment.3:Diet.f_limited diet                            | NA         |
| trait_pigment.3:Diet.f_mixed diet                              | 1.8765185  |
| trait_pigment.3:Diet.f_phyto diet                              | 4.1743878  |
| trait_wetwteyes:Diet.f_limited diet                            | NA         |
| trait_wetwteyes:Diet.f_mixed diet                              | -0.4814127 |
| trait_wetwteyes:Diet.f_phyto diet                              | 0.5448324  |
| trait_pigment.3:Temp.f_Cold                                    | NA         |
| trait_pigment.3:Temp.f_Warm                                    | 3.4762492  |

```

trait_wetwteyes:Temp.f_Cold          NA
trait_wetwteyes:Temp.f_Warm          2.0424007
trait_pigment.3:Age.f_1 month        NA
trait_pigment.3:Age.f_4 months       0.4353662
trait_pigment.3:Age.f_7 months       -1.1278869
trait_wetwteyes:Age.f_1 month        NA
trait_wetwteyes:Age.f_4 months       0.2153657
trait_wetwteyes:Age.f_7 months       -0.1862226
trait_pigment.3                      9.6169633
trait_wetwteyes                      2.4317927
>
> vc <- ((summary(asreml.mv))$varcomp)[[1]]
>
> vc[3]/((vc[2]*vc[4])^0.5)
[1] 0.02274260
>
> asreml.mv <- asreml(fixed = cbind(pigment.3,wetwteyes) ~ trait +
+   trait:Age.f+trait:Temp.f+
+   trait:Age.f:Temp.f+trait:Diet.f+trait:Diet.f:Temp.f,
+   rcov= ~ units:us(trait), data=data.df, na.method.Y = "exclude",
+   na.method.X = "exclude", maxiter=30)

```

asreml(): 2.00 Library: 2.00bb Run: Thu Oct 09 09:42:21 2008  
 Licensed to: Australian Antarctic Division  
 Serial Number: 402061977 Expires: 30-sep-2009 (356 days)

Equations: 42 (42 dense)  
 Initial update shrinkage factor: 0.316  
 22 singularities detected in design matrix

| LogLik    | S2     | DF  |          |
|-----------|--------|-----|----------|
| 752.9170  | 1.0000 | 504 | 09:42:21 |
| 1052.6507 | 1.0000 | 504 | 09:42:21 |
| 1321.8611 | 1.0000 | 504 | 09:42:21 |
| 1538.5462 | 1.0000 | 504 | 09:42:21 |
| 1617.6696 | 1.0000 | 504 | 09:42:21 |
| 1638.2173 | 1.0000 | 504 | 09:42:21 |
| 1640.7847 | 1.0000 | 504 | 09:42:21 |
| 1640.8529 | 1.0000 | 504 | 09:42:21 |
| 1640.8530 | 1.0000 | 504 | 09:42:21 |

Finished on: Thu Oct 09 09:42:21 2008

LogLikelihood Converged

```

>
>
>
> anova(asreml.mv)
Wald tests for fixed effects

```

Response: cbind(pigment.3, wetwteyes)

Terms added sequentially; adjusted for those above

|                     | Df | Sum of Sq | Wald statistic | Pr(Chisq)     |
|---------------------|----|-----------|----------------|---------------|
| trait               | 2  | 2081.53   | 2081.53        | < 2.2e-16 *** |
| trait:Age.f         | 4  | 45.13     | 45.13          | 3.736e-09 *** |
| trait:Temp.f        | 2  | 4.89      | 4.89           | 0.08682 .     |
| trait:Diet.f        | 4  | 6.24      | 6.24           | 0.18185       |
| trait:Age.f:Temp.f  | 4  | 44.77     | 44.77          | 4.447e-09 *** |
| trait:Temp.f:Diet.f | 4  | 38.85     | 38.85          | 7.482e-08 *** |
| residual (MS)       |    | 1.00      |                |               |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

> (summary(asreml.mv))\$varcomp

|                             | gamma        | component    | std.error    | z.ratio    |
|-----------------------------|--------------|--------------|--------------|------------|
| R!variance                  | 1.000000e+00 | 1.000000e+00 | NA           | NA         |
| R!trait.pigment.3:pigment.3 | 1.912588e-03 | 1.912588e-03 | 1.703866e-04 | 11.2249898 |
| R!trait.wetwteyes:pigment.3 | 1.467332e-05 | 1.467332e-05 | 3.002708e-05 | 0.4886695  |
| R!trait.wetwteyes:wetwteyes | 1.186845e-04 | 1.186845e-04 | 1.057326e-05 | 11.2249725 |

constraint

|                             | Fixed         |
|-----------------------------|---------------|
| R!variance                  |               |
| R!trait.pigment.3:pigment.3 | Unconstrained |
| R!trait.wetwteyes:pigment.3 | Unconstrained |
| R!trait.wetwteyes:wetwteyes | Unconstrained |

> (summary(asreml.mv))\$coef.fixed

|                                                 | solution      | std error   |
|-------------------------------------------------|---------------|-------------|
| trait_pigment.3:Temp.f_Cold:Diet.f_limited diet | 0.000000e+00  | NA          |
| trait_pigment.3:Temp.f_Cold:Diet.f_mixed diet   | 0.000000e+00  | NA          |
| trait_pigment.3:Temp.f_Cold:Diet.f_phyto diet   | 0.000000e+00  | NA          |
| trait_pigment.3:Temp.f_Warm:Diet.f_limited diet | 0.000000e+00  | NA          |
| trait_pigment.3:Temp.f_Warm:Diet.f_mixed diet   | -4.373863e-02 | 0.014530832 |
| trait_pigment.3:Temp.f_Warm:Diet.f_phyto diet   | -8.338813e-02 | 0.014280546 |
| trait_wetwteyes:Temp.f_Cold:Diet.f_limited diet | 0.000000e+00  | NA          |
| trait_wetwteyes:Temp.f_Cold:Diet.f_mixed diet   | 0.000000e+00  | NA          |
| trait_wetwteyes:Temp.f_Cold:Diet.f_phyto diet   | 0.000000e+00  | NA          |
| trait_wetwteyes:Temp.f_Warm:Diet.f_limited diet | 0.000000e+00  | NA          |
| trait_wetwteyes:Temp.f_Warm:Diet.f_mixed diet   | 3.370627e-05  | 0.003619737 |
| trait_wetwteyes:Temp.f_Warm:Diet.f_phyto diet   | -6.218783e-03 | 0.003557389 |
| trait_pigment.3:Age.f_1 month:Temp.f_Cold       | 0.000000e+00  | NA          |
| trait_pigment.3:Age.f_1 month:Temp.f_Warm       | 0.000000e+00  | NA          |
| trait_pigment.3:Age.f_4 months:Temp.f_Cold      | 0.000000e+00  | NA          |
| trait_pigment.3:Age.f_4 months:Temp.f_Warm      | 8.279127e-03  | 0.012376712 |
| trait_pigment.3:Age.f_7 months:Temp.f_Cold      | 0.000000e+00  | NA          |
| trait_pigment.3:Age.f_7 months:Temp.f_Warm      | -7.900547e-02 | 0.015225221 |
| trait_wetwteyes:Age.f_1 month:Temp.f_Cold       | 0.000000e+00  | NA          |

|                                            |               |             |
|--------------------------------------------|---------------|-------------|
| trait_wetwteyes:Age.f_1 month:Temp.f_Warm  | 0.000000e+00  | NA          |
| trait_wetwteyes:Age.f_4 months:Temp.f_Cold | 0.000000e+00  | NA          |
| trait_wetwteyes:Age.f_4 months:Temp.f_Warm | -3.222722e-03 | 0.003083130 |
| trait_wetwteyes:Age.f_7 months:Temp.f_Cold | 0.000000e+00  | NA          |
| trait_wetwteyes:Age.f_7 months:Temp.f_Warm | -4.380136e-03 | 0.003792714 |
| trait_pigment.3:Diet.f_limited diet        | 0.000000e+00  | NA          |
| trait_pigment.3:Diet.f_mixed diet          | 1.853315e-02  | 0.009967788 |
| trait_pigment.3:Diet.f_phyto diet          | 5.133918e-02  | 0.010064035 |
| trait_wetwteyes:Diet.f_limited diet        | 0.000000e+00  | NA          |
| trait_wetwteyes:Diet.f_mixed diet          | -2.145867e-03 | 0.002483049 |
| trait_wetwteyes:Diet.f_phyto diet          | 2.169476e-03  | 0.002507025 |
| trait_pigment.3:Temp.f_Cold                | 0.000000e+00  | NA          |
| trait_pigment.3:Temp.f_Warm                | 4.829076e-02  | 0.011749263 |
| trait_wetwteyes:Temp.f_Cold                | 0.000000e+00  | NA          |
| trait_wetwteyes:Temp.f_Warm                | 5.268359e-03  | 0.002926828 |
| trait_pigment.3:Age.f_1 month              | 0.000000e+00  | NA          |
| trait_pigment.3:Age.f_4 months             | -2.470420e-02 | 0.008461291 |
| trait_pigment.3:Age.f_7 months             | 6.103219e-02  | 0.009967788 |
| trait_wetwteyes:Age.f_1 month              | 0.000000e+00  | NA          |
| trait_wetwteyes:Age.f_4 months             | 8.409274e-05  | 0.002107770 |
| trait_wetwteyes:Age.f_7 months             | 1.592117e-03  | 0.002483049 |
| trait_pigment.3                            | 9.567527e-02  | 0.008276994 |
| trait_wetwteyes                            | 6.030464e-03  | 0.002061860 |

#### z ratio

|                                                 |              |
|-------------------------------------------------|--------------|
| trait_pigment.3:Temp.f_Cold:Diet.f_limited diet | NA           |
| trait_pigment.3:Temp.f_Cold:Diet.f_mixed diet   | NA           |
| trait_pigment.3:Temp.f_Cold:Diet.f_phyto diet   | NA           |
| trait_pigment.3:Temp.f_Warm:Diet.f_limited diet | NA           |
| trait_pigment.3:Temp.f_Warm:Diet.f_mixed diet   | -3.010056858 |
| trait_pigment.3:Temp.f_Warm:Diet.f_phyto diet   | -5.839281614 |
| trait_wetwteyes:Temp.f_Cold:Diet.f_limited diet | NA           |
| trait_wetwteyes:Temp.f_Cold:Diet.f_mixed diet   | NA           |
| trait_wetwteyes:Temp.f_Cold:Diet.f_phyto diet   | NA           |
| trait_wetwteyes:Temp.f_Warm:Diet.f_limited diet | NA           |
| trait_wetwteyes:Temp.f_Warm:Diet.f_mixed diet   | 0.009311801  |
| trait_wetwteyes:Temp.f_Warm:Diet.f_phyto diet   | -1.748131432 |
| trait_pigment.3:Age.f_1 month:Temp.f_Cold       | NA           |
| trait_pigment.3:Age.f_1 month:Temp.f_Warm       | NA           |
| trait_pigment.3:Age.f_4 months:Temp.f_Cold      | NA           |
| trait_pigment.3:Age.f_4 months:Temp.f_Warm      | 0.668927839  |
| trait_pigment.3:Age.f_7 months:Temp.f_Cold      | NA           |
| trait_pigment.3:Age.f_7 months:Temp.f_Warm      | -5.189118268 |
| trait_wetwteyes:Age.f_1 month:Temp.f_Cold       | NA           |
| trait_wetwteyes:Age.f_1 month:Temp.f_Warm       | NA           |
| trait_wetwteyes:Age.f_4 months:Temp.f_Cold      | NA           |
| trait_wetwteyes:Age.f_4 months:Temp.f_Warm      | -1.045275991 |
| trait_wetwteyes:Age.f_7 months:Temp.f_Cold      | NA           |
| trait_wetwteyes:Age.f_7 months:Temp.f_Warm      | -1.154881668 |
| trait_pigment.3:Diet.f_limited diet             | NA           |
| trait_pigment.3:Diet.f_mixed diet               | 1.859304291  |

```

trait_pigment.3:Diet.f_phyto diet      5.101251854
trait_wetwteyes:Diet.f_limited diet      NA
trait_wetwteyes:Diet.f_mixed diet      -0.864206388
trait_wetwteyes:Diet.f_phyto diet      0.865358640
trait_pigment.3:Temp.f_Cold      NA
trait_pigment.3:Temp.f_Warm      4.110109812
trait_wetwteyes:Temp.f_Cold      NA
trait_wetwteyes:Temp.f_Warm      1.800023655
trait_pigment.3:Age.f_1 month      NA
trait_pigment.3:Age.f_4 months      -2.919672827
trait_pigment.3:Age.f_7 months      6.122942663
trait_wetwteyes:Age.f_1 month      NA
trait_wetwteyes:Age.f_4 months      0.039896550
trait_wetwteyes:Age.f_7 months      0.641194290
trait_pigment.3      11.559180851
trait_wetwteyes      2.924768759
>
> vc <- ((summary(asreml.mv))$varcomp)[[1]]
>
> vc[3]/((vc[2]*vc[4])^0.5)
[1] 0.03079786
>
> asreml.mv <- asreml(fixed = cbind(CL,wetwteyes) ~ trait + trait:Age.f,
+   rcov= ~ units:us(trait), data=data.df, na.method.Y = "exclude",
+   na.method.X = "exclude", maxiter=30)

```

asreml(): 2.00 Library: 2.00bb Run: Thu Oct 09 09:42:38 2008  
 Licensed to: Australian Antarctic Division  
 Serial Number: 402061977 Expires: 30-sep-2009 (356 days)

Equations: 8 (8 dense)  
 Initial update shrinkage factor: 0.316  
 2 singularities detected in design matrix

```

LogLik      S2      DF
-179.9145    1.0000  518 09:42:38 (3 component(s) constrained)
Warning: 2 1 NonPosDef US matrix modified
-40.8798     1.0000  518 09:42:38 (3 component(s) constrained)
Warning: 2 1 NonPosDef US matrix modified
 14.0394     1.0000  518 09:42:38 (3 component(s) constrained)
Warning: Since fault 3 occurred during the last iteration results reported may be
erroneous
ABORT
Fault 3 Negative Sum of Squares
Finished on: Thu Oct 09 09:42:38 2008

```

Error: Abnormal termination  
 Negative Sum of Squares  
 >





## Appendix V: Nutritional profile of *Schizochytrium* sp. (Algamac-2000®)

| Proximate Analysis     | % weight       |
|------------------------|----------------|
| Protein                | 20.2           |
| Fat                    | 38.1           |
| Carbohydrate           | 17.1           |
| Ash                    | 20.4           |
| Moisture               | 4.2            |
| Calories               | 492.0          |
| Sterols & other        | mg/100g        |
| Beta-Sitosterol        | 45.1           |
| Campesterol            | 82.4           |
| Cholesterol            | 213.0          |
| Stigmasterol           | 225.0          |
| Lecithin               | 392            |
| Lutein                 | <0.12          |
| Fatty Acid profile     | %w/w Total FAs |
| Myristate              | 16.30          |
| Palmitate              | 42.20          |
| Palmitoleate           | 1.75           |
| Stearate               | 1.32           |
| Oleate                 | 0.11           |
| Eicosatrienoic (ETA)   | 0.11           |
| Eicosapentaenoic (EPA) | 0.54           |
| Docosapentaenoic (DPA) | 10.67          |
| Docosahexaenoic (DHA)  | 27.00          |
| Vitamins               | (mg/100g)      |
| Biotin                 | 131.00         |
| Choline                | 83.10          |
| Folic Acid             | 350.00         |
| Inositol               | 83.00          |
| Niacin                 | 11.80          |
| Pantothenic Acid       | 4.95           |
| Pyridoxine             | 0.319          |
| Riboflavin             | 1.64           |
| Thiamine               | 0.38           |
| Vitamin B12            | 55.10          |
| Vitamin A (IU/100g)    | <100           |
| Vitamin D (IU/100g)    | 457.00         |
| Vitamin E (IU/100g)    | 12.00          |

Source: Aquafauna Bio-Marine, Inc.